## *Remarks*

Reconsideration of this Application is respectfully requested.

Claims 75-78, 81-84, 86-103, 105-107 and 109-145 are pending in the application, with claims 75, 102, 103 and 125 being the independent claims.

Based on the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding rejections and that they be withdrawn.

### *I. Claim Rejections Under 35 U.S.C. § 112, First Paragraph*

#### *A. Written Description*

Claims 75-78, 81-84, 86-101, 103, 105-107 and 109-145 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. *See* Paper No. 30, page 3. Applicants respectfully traverse this rejection for the reasons set forth in Applicants' Amendment and Reply filed November 4, 2002, pages 15-17, and in Applicants' Supplemental Reply filed March 31, 2003, pages 2-7.

To satisfy the written description requirement of 35 U.S.C. § 112, first paragraph, an Applicant must convey with reasonable clarity to those skilled in the art that, as of the effective filing date, the Applicant was in possession of the invention. *See Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1560, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991). As recently articulated by the Federal Circuit, "[t]he purpose of the written description requirement is

to prevent an applicant from later asserting that he invented that which he did not." *See Amgen Inc. v. Hoechst Marion Roussel Inc.*, 65 USPQ2d 1385, 1397 (Fed. Cir. 2003).

The present claims are directed to, or involve the use of, nucleic acid molecules which comprise a first open reading frame which encodes a non-cytopathic, temperature-sensitive alphaviral replicase. The claims specify that non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the non-structural proteins of the replicase. A person of ordinary skill in the art, in view of the specification, would conclude that Applicants invented the subject matter encompassed by the claims. Thus, the written description requirement of 35 U.S.C. § 112, first paragraph, is fully satisfied.

The specification clearly indicates that Applicants were in possession of the invention insofar as it encompasses or involves the use of a nucleic acid molecule that encodes a non-cytopathic, temperature-sensitive alphaviral replicase, wherein non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the non-structural proteins of the replicase. For example, the Specification provides the following summary of the invention:

> The present invention provides compositions and methods for regulated expression of proteins or untranslated RNA molecules in recombinant host cells. More specifically, the present invention provides polynucleotides and methods which allow precise regulation of the amount of specific RNA molecules produced in stably transfected recombinant host cells. This precise regulation results from the use of a temperature-sensitive RNA-dependent RNA polymerase (*i.e.*, a replicase) which only replicates RNA molecules, to form new RNA molecules, at permissive temperature.

Specification at page 6, lines 17-24. It is further noted that:

> When using alphavirus replicase proteins, in most instances, it is desirable to convert the cytopathic phenotype of the replicase protein to a non-cytopathic phenotype. Preferred mutations which confer such a phenotype are in the nsp2 gene (*e.g.*, the proline residue at position 726 is replaced with a serine residue). Mutations are known in the art which render the replicase protein non-cytopathic (Weiss *et al.*, *J. Virol. 33*:463-474 (1980); Dryga *et al.*, *Virology 228*:74-83 (1997)). These mutations may be introduced by a number of means, including site directed mutagenesis.
>
> As noted above, when a non-cytopathic Sindbis virus replicase is used in the practice of the invention, a mutation may be introduced in the nsp2 gene. One such mutation results from the exchange of the proline residue at position 726 to another of the 20 natural occurring amino acids, such as a serine (abbreviated as "Pro 726 Ser"). *Alternatively, any other mutation rendering the replicase molecule non-cytopathic is within the scope of the invention.* The creation and the identification of mutations which render the Sindbis replicase non-cytopathic are described in more detail elsewhere (Weiss *et al.*, *J. Virol. 33*:463-474 (1980); Dryga *et al.*, *Virology 228*:74-83 (1997); patent application WO 97/38087). Further, methods for inducing such mutations are known in the art (*see, e.g.*, Sambrook, J. *et al.*, eds., MOLECULAR CLONING, A LABORATORY MANUAL, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel, F. *et al.*, eds., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John H. Wiley & Sons, Inc. (1997)).

Specification at page 21, lines 23-30 (emphasis added).

With respect to temperature sensitivity, it is noted that:

> Temperature sensitivity (*ts*) may be conferred, for example, by the introduction of a mutation in the nsp4 gene of the replicase. Preferably, mutations which confer a temperature-sensitive phenotype upon replicase activities are in a protein in complementation group F (Lemm *et al.*, *J. Virol. 64*:3001-3011 (1990)). For example, a temperature-sensitive phenotype may be conferred by changing Gly 153 of nsp4 to Glu. *Additionally, any other mutation which renders replicase activity temperature-sensitive can be used in the practice of the invention.* Methods for creating and identifying new temperature-sensitive mutants are described by Pfefferkorn (Burge and Pfefferkorn, *Virol.*

> *30*:204-213(1966); Burge and Pfefferkorn, *Virol. 30*:214-223 (1966)). Further, any method useful for producing and identifying *ts* mutants which allow for the temperature-sensitive regulation of replicase activity can be employed to generate and isolate such mutants.

Specification at page 22, lines 13-25. Thus, it is clear from the specification that the invention encompasses nucleic acid molecules that encode non-cytopathic, temperature-sensitive alphaviral replicases. It is also clear from the specification that the properties of non-cytopathicity and temperature sensitivity can be conferred upon the replicases by *any mutation(s)* that render the replicase non-cytopathic and temperature sensitive. Methods for obtaining such mutations were well known to persons of ordinary skill in the art as of the effective filing date of the application. *See* discussion below.

The specification provides a working example of a nucleic acid molecule that encodes a non-cytopathic, temperature-sensitive alphaviral replicase. The construction of this nucleic acid molecule, designated pCYTts, is described in the specification at page 41, line 2, through page 43, line 8 (Example 1). This exemplary nucleic acid molecule encodes a Sindbis virus replicase containing a Pro726Ser mutation in nsp2 and a Gly153Glu mutation in nsp4. The detailed description of this exemplary embodiment reinforces the conclusion that Applicants were in possession of the invention insofar as it encompasses or involves the use of a nucleic acid molecule that encodes a non-cytopathic, temperature-sensitive alphaviral replicase.

According to the Examiner, "[t]he central issue in this analysis is whether Applicant has disclosed a number of species which is representative of the claimed genus." *See* Paper No. 30, page 4. The Examiner has cited the Federal Register, Vol. 64, No. 244, pp. 71427-71440 to support this assertion. The written description guidelines cited by the Examiner,

however, indicate that there are other ways to satisfy the written description requirement besides disclosure of a representative number of species. According to the guidelines, the written description requirement may also be satisfied:

> by disclosure of relevant, identifying characteristics, *i.e.*, structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, *or by a combination of such identifying characteristics*, sufficient to show that the Applicant was in possession of the claimed genus.

*See id.* (emphasis added).

Applicants have disclosed: (a) the functional characteristics of the nucleic acid molecules included in the invention (*i.e.*, that they encode a non-cytopathic, temperature sensitive alphaviral replicase); and (b) the structural characteristics of the nucleic acid molecules included in the invention (*i.e.*, that non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of the replicase[1]). It was well known in the art that mutations in the genes encoding the nonstructural proteins of alphaviral replicases result in the phenotypes of non-cytopathicity and/or temperature sensitivity. *See, e.g.*, Exhibits 1-12 submitted with the Supplemental Reply filed on March 31, 2003. Furthermore, mutations that render an alphaviral replicase non-cytopathic and temperature sensitive could have easily been obtained by persons of ordinary skill in the art. The ability of those skilled in the art to obtain non-cytopathic, temperature-sensitive alphaviral replicases is described in the Declaration of Dr. Sondra Schlesinger Under 37 C.F.R. § 1.132, submitted with the Supplemental Reply filed on

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[1]For claims 137-145, even more detailed structural definition is provided since these claims specify that non-cytopathicity is conferred by one or more mutations in the nsp2 gene and/or that temperature sensitivity is conferred by one or more mutations in the nsp4 gene.

March 31, 2003, and is also addressed in the response to the Enablement rejection below. Thus, there is a known and disclosed correlation between function and structure. Based on the guidelines cited by the Examiner, the written description requirement is satisfied for the claimed subject matter.

The Examiner has based the written description rejection on the absence of multiple working examples of non-cytopathic, temperature-sensitive alphaviral replicases. As indicated above, satisfaction of the written description requirement does not necessarily require the disclosure of multiple working examples. There are other factors that must be taken into consideration such as, *e.g.*, the disclosure of structural and functional characteristics and the correlation between structure and function. When such factors are considered in the context of the present invention, it must be concluded that the written description requirement is satisfied.

According to the Examiner, "the specification has failed to disclose what mutations are required to render any other RNA-dependent RNA polymerase both temperature sensitive and non-cytopathic, or what other mutations could confer this phenotype on the Sindbis virus polymerase." *See* Paper No. 30, page 4. As mentioned above, it was known that mutations in the nonstructural protein genes of alphaviruses produce non-cytopathicity and/or temperature sensitivity, and that a person of ordinary skill in the art, in view of the present specification, could have easily obtained nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases. Satisfaction of the written description requirement therefore does not require that all possible mutations that confer these phenotypes be recited in the specification.

The Examiner asserted that, aside from the Sindbis virus nsp2 P726S mutation, which is shown to cause non-cytopathicity, "Applicant's response filed 3/31/03 does not disclose any [other mutations which cause non-cytopathicity]." *See* Paper No. 30, page 8. Applicants respectfully point to Exhibit 12, *i.e.*, Perri *et al.*, *J. Virol. 74*:9802-9807 (2000), submitted with the Supplemental Reply filed on March 31, 2003. Perri describes the isolation of several non-cytopathic Sindbis and Semliki Forest virus replicase mutants. The amino acid changes found in the mutants are described in Perri at page 9804. In addition, Applicants submit herewith two additional references that describe the production of nucleic acid molecules encoding non-cytopathic, temperature sensitive Semliki Forest virus replicases. *See* Lundstrom *et al.*, *Histochem. Cell. Biol. 115*:83-91 (2001) (copy submitted herewith as Exhibit A), and Lundstrom *et al.*, *Mol. Ther. 7*:202-209 (2003) (copy submitted herewith as Exhibit B). The replicases described in these references were obtained using PCR-mediated site-directed mutagenesis. Although these references were published after the effective filing date of the present application, the techniques and materials used to produce the mutants were known and available to persons of ordinary skill in the art well before the effective filing date. It also appears that the information that was used by Perri and Lundstrom to select the particular mutations described in the references would have also been available to persons of ordinary skill in the art prior to the effective filing date of the present application. Thus, the Perri and Lundstrom references support Applicants' position that nucleic acid molecules encoding non-cytopathic, temperature-sensitive replicases that fall within the scope of the present claims could have easily been obtained by persons of ordinary skill in the art.

The working example in the specification involves a Sindbis virus non-cytopathic, temperature-sensitive replicase. As discussed in Applicants' previous reply, there is a high degree of sequence homology among the nsPs from various alphaviruses. *See* Applicants' Supplemental Reply filed March 31, 2003, pages 5-6, and Exhibits 13-18 submitted therewith. Thus, a person of ordinary skill in the art could have easily produced non-cytopathic, temperature-sensitive replicases from other alphaviruses using the Sinbis virus mutations described in the present specification as a guide. The present application, as well as other information available in the art, would have pointed the skilled artisan to the amino acids (or regions) in other alphavirus replicases that, when mutated, would likely produce non-cytopathic and temperature sensitive phenotypes.

In summary, the present specification clearly indicates that the invention includes nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases, and that non-cytopathicity and temperature sensitivity can be conferred by any mutations that result in these phenotypes. The claims specify that non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of the replicase and therefore provide a structural and functional definition of the invention. It was known in the art that mutations in the nonstructural protein genes cause temperature sensitivity and/or non-cytopathicity in alphaviral replicases. In addition, mutations in genes encoding the nonstructural proteins of an alphaviral replicase and that confer non-cytopathicity and temperature sensitivity could have easily been identified and produced by persons of ordinary skill in the art. Thus, there is a known and disclosed correlation between structure and function. A person of ordinary skill in the art would therefore conclude that Applicants were in possession of the subject matter encompassed by

the claims. Applicants respectfully request that the written description rejection of claims 75-78, 81-84, 86-101, 103, 105-107 and 109-145 be reconsidered and withdrawn.

***B. Enablement***

Claims 75-79, 81-84, 86-101, 103, 105-107 and 109-136 were rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. According to the Examiner, "the specification, while being enabling for a DNA molecule encoding the Sindbis virus non-cytopathic, temperature-sensitive alphaviral replicase with P726S nsp2 and G153E nsp4 mutations, does not reasonably provide enablement for DNA molecules encoding any other alphaviral non-cytopathic, temperature-sensitive alphavirus replicases." *See* Paper No. 30, page 10. Applicants respectfully traverse this rejection.

The Enablement rejection is based on the Examiner's assertion that it is difficult to predict the relationship between nucleic acid mutations and protein function. As noted in Applicants' Supplemental Reply, filed on March 31, 2003, the construction and selection of nucleic acid molecules that encode temperature-sensitive, non-cytopathic alphavirus replicases would not require one of ordinary skill in the art to make predictions regarding the effects of mutations on protein function. Rather, the creation of temperature-sensitive, non-cytopathic alphaviral replicases requires only that a skilled artisan engage, for example, in the mutagenesis of a nucleic acid encoding an alphaviral replicase and then screen for those molecules which possess the desired phenotypes (temperature sensitivity and non-cytopathicity). Such screening would not be regarded as undue experimentation. Applicants' assertion that the production of non-cytopathic, temperature sensitive alphaviral replicases could have been accomplished using only routine techniques is supported by the

Declaration of Professor Sondra Schlesinger Under 37 C.F.R. § 1.132, submitted with Applicants' Supplemental Reply, filed on March 31, 2003.

The Examiner dismissed the Declaration of Dr. Schlesinger because, according to the Examiner, "the Declaration provides no factual evidence whatsoever, and is only a statement of Declarants [sic: Declarant's] beliefs." Paper No. 30, page 15. Applicants respectfully request that the Examiner reconsider the Declaration. Applicants note that the Declaration of Dr. Schlesinger simply confirms an approach to identifying mutations that produce specific desired phenotypes that was well known in the art as of the effective filing date of the present invention. The approach described by Dr. Schlesinger in paragraph 5 of the Declaration is nothing more than a basic genetic screening method known and practiced for decades prior to the present invention. *See, e.g.*, Sambrook *et al.*, "Creating Many Mutations in a Defined Segment of DNA," in *Molecular Cloning, A Laboratory Manual*, Sambrook *et al.*, eds., Cold Spring Harbor Laboratory Press, pp. 15.95-15.108 (1989) (copy submitted with the Amendment and Reply Under 37 C.F.R. § 1.116, filed on November 4, 2002). In fact, a review of the scientific literature demonstrates that random mutagenesis and genetic screening approaches have been used for many years to identify alphaviral mutants. *See, e.g.*, Keränen and Kääriäinen, *Acta Path. Microbiol. Scand. Sect. B, 82*:810-820 (1974) (copy submitted herewith as Exhibit C), and Hearne *et al.*, *J. Gen. Virol. 68*:107-113 (1987) (copy submitted herewith as Exhibit D). The Examiner has not explained why such a basic method for obtaining mutants with a desired phenotype would not have been used in the context of the present invention. Moreover, it is unclear what particular aspects of the Declaration the Examiner finds unconvincing.

In addition, under 35 U.S.C. § 112, first paragraph, the Examiner has the initial burden of presenting specific evidence to indicate that making and/or using the subject matter of the claims would require undue experimentation. *See In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). The only arguments the Examiner has made with respect to enablement relate to whether one of ordinary skill in the art could predict the functional consequences of mutations. Applicants submit that such arguments are irrelevant to assessing the enablement of the present invention because the production of non-cytopathic, temperature sensitive alphaviral replicases would not require one of ordinary skill in the art to predict the effects of particular mutations on protein function. Rather, one of ordinary skill in the art would be expected to use a genetic screening and selection approach to make and identify non-cytopathic, temperature sensitive alphaviral replicases. The Declaration of Dr. Schlesinger supports this position, as do the numerous examples in the scientific literature that describe the successful production of non-cytopathic and/or temperature sensitive alphaviral replicases. *See, e.g.*, Exhibits 1-12 submitted with the Supplemental Reply filed on March 31, 2003, and Exhibits A and B submitted herewith.

The Examiner apparently disagrees on two levels with Applicants' assertion. First, the Examiner apparently does not believe that a person of ordinary skill in the art would use a genetic screening and selection approach to obtain nucleic acid molecules that encode non-cytopathic, temperature sensitive alphaviral replicases. Second, the Examiner apparently does not believe that such a method would successfully result in the production of nucleic acid molecules that encode non-cytopathic, temperature sensitive alphaviral replicases. The Examiner, however, has not presented any evidence to support either of these positions. To Establish a *prima facie* case of non-enablement, "it is incumbent upon

the Patent Office. . . to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." *See In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971) (emphasis in original). Thus, regardless of whether the Examiner chooses to believe the Declaration of Dr. Schlesinger, the Examiner has not met the burden of establishing a *prima facie* case of non-enablement.

In summary, the present invention does not require the ability of one skilled in the art to predict the functional consequences of mutations. A person of ordinary skill in the art, in order to obtain nucleic acid molecules that encode non-cytopathic, temperature sensitive alphaviral replicases, would have used a genetic screening and selection approach, as noted in the Declaration of Professor Sondra Schlesinger Under 37 C.F.R. § 1.132. Such an approach would not have required undue experimentation. The Examiner has not presented any evidence or scientifically sound argument to explain why a person of ordinary skill in the art would have needed to predict the functional consequences of mutations in order to make and use the subject matter of the present invention. The Examiner has not presented any evidence or scientifically sound argument to indicate that a person of ordinary skill in the art would *not* have used a genetic selection and screening approach to obtain nucleic acid molecules that encode non-cytopathic, temperature sensitive alphaviral replicases. The Examiner has not presented any evidence or scientifically sound argument to indicate that a genetic selection and screening approach would not have resulted in the identification of nucleic acid molecules that encode non-cytopathic, temperature sensitive alphaviral replicases, or that such an approach would have involved undue experimentation. Thus, the present invention is fully enabled and the Examiner has not established a *prima facie* case

of non-enablement. Applicants therefore respectfully request that the enablement rejection of claims 75-79, 81-84, 86-101, 103, 105-107 and 109-136 be reconsidered and withdrawn.

## *Conclusion*

All of the stated grounds of rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Reply is respectfully requested.

Respectfully submitted,

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ORIGINAL PAPER

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# Novel mutant Semliki Forest virus vectors: gene expression and localization studies in neuronal cells

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**Abstract** Semliki Forest virus vectors (SFV) are suitable for high-level transgene expression in neuronal tissue, both in vitro and in vivo. Cortical and hippocampal primary neurons in culture are efficiently infected resulting in 75–95% of GFP-positive cells, and injection of SFV vectors into hippocampal slice cultures revealed a highly neuron-specific expression pattern with more than 90% of the infected cells being neurons. Here, we present novel SFV vector mutants and describe their infection patterns obtained in cultures of baby hamster kidney (BHK) cells, dissociated hippocampal neurons, and organotypic hippocampal slices. A less cytotoxic vector SFV(PD), carrying two point mutations in the nsP2 gene, showed much higher GFP expression levels in primary hippocampal neurons compared to the wild-type SFV vector. A triple mutant vector SFV($PDE_{153}$) demonstrated a temperature-sensitive phenotype in both BHK cells and primary neurons. In hippocampal slices cultured at 36°C, SFV($PDE_{153}$) showed a remarkably higher (ca 250-fold) preference for expression in interneurons rather than in pyramidal cells as compared to wild-type SFV. The quadruple mutant SFV(PDTE) led to substantially increased and prolonged GFP expression in primary neurons. Relative to SFV($PDE_{153}$), a more pronounced temperature-sensitive phenotype was found resulting in no virus production and no GFP expression at the non-permissive temperature (36–37°C) in BHK cells, in dissociated neurons, and in organotypic hippocampal slices. The described novel SFV vectors will be useful for several specific applications in neurobiology.

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**Keywords** Semliki Forest virus · GFP expression · Temperature-sensitive mutants · Hippocampal slice cultures · Dissociated hippocampal neurons

## Introduction

Gene delivery to neuronal cells has been a real challenge. Most of the transfection methods developed have been relatively inefficient. Much attention has therefore been paid to employing viruses, known to efficiently infect nerve cells, for this task. However, even viruses have clear restrictions in their capacity to infect non-dividing cells like neurons. For example, retroviruses are not able to transduce neuronal cells (Miller et al. 1990). Other viruses like adenoviruses do transduce neurons quite efficiently (see, for example, Ehrengruber et al. 1997), but show preferential infection of glial cells when these are growing in close connection with neurons (Ehrengruber et al., manuscript in preparation). Adenovirus vectors are therefore not particularly suitable for gene expression studies in primary neuron cultures with a feeder layer of glial cells. Similarly, studies in neurons of hippocampal slice cultures and in vivo are not suitable with adenovirus vectors. Other viruses like herpes simplex virus (Fotaki et al. 1997) and adeno-associated virus (Paterna et al. 2000) have shown efficient infection of neurons. These vectors are highly efficient for long-term expression, but their disadvantage is that the virus production is rather labor-intensive and time-consuming.

Alphavirus vectors are known for their rapid and simple generation of high-titer replication-deficient particles. Expression systems have been developed for both Semliki Forest virus (SFV; Liljeström and Garoff 1991) and Sindbis virus (Xiong et al. 1989). The most commonly used system is based on two plasmid vectors: (1) an expression vector containing the non-structural viral genes (required for RNA replication) and the foreign gene(s) of interest inserted behind the strong subgenomic 26S promoter and (2) a helper vector containing the viral structural genes (capsid and membrane protein genes) re-

quired for the assembly of recombinant alphavirus particles. The second-generation SFV vector, pSFV-Helper2, has as an additional safety feature; three point mutations introduced in the p62 precursor near the cleavage of the E2 and E3 membrane proteins (Berglund et al. 1993). This leads to the production of conditionally infectious SFV particles and will reduce the generation of replication-competent particles through homologous recombination to a level below detection. SFV vectors exhibit a broad host range and have been shown to infect a variety of mammalian cell lines and primary cell cultures (Lundstrom 1999). Extremely high levels of recombinant protein expression of topologically different proteins have been obtained. The infection rate of primary rat hippocampal neurons has been remarkably high (75–95%; Lundstrom et al. 1998). Furthermore, injection of SFV-GFP into hippocampal slice cultures demonstrated a high preference for neuronal infection, as more than 90% of the GFP-positive cells were of neuronal origin (Ehrengruber et al. 1999). Moreover, stereotactic injections of SFV-LacZ virus into amygdala and striatum of rat brain resulted in highly neuron-specific expression of β-galactosidase (Lundstrom et al. 1999a).

We have here studied the expression of novel non-cytopathogenic and temperature-sensitive SFV mutants in baby hamster kidney (BHK) cells, in cultures of dissociated rat hippocampal neurons, as well as in organotypic rat hippocampal slices. These novel SFV vectors showed superior expression levels compared to the wild-type SFV. Interestingly, in hippocampal slice cultures, one of the temperature-sensitive triple mutants had a different expression pattern in interneurons and pyramidal cells at the non-permissive temperature compared to the wild-type SFV. On the other hand, the quadruple mutant showed absolutely no transgene expression at the non-permissive temperature in all culture systems tested.

## Materials and methods

### Cell cultures

BHK cells were cultured in a mixture of Dulbecco's modified F-12 medium (Gibco BRL, Gaithersburg, Md., USA) and Iscove's modified Dulbecco's medium (Gibco BRL), 1:1 mixture, supplemented with glutamine and 10% fetal calf serum. Rat primary hippocampal neurons were cultured from rat embryos (age E17; strain ROROspf120; BRL, Fullinsdorf, Switzerland) as earlier described (Lundstrom et al. 1998).

### Construction of mutant SFV vectors

The construct pSFV(PD)-GFP has been described earlier (Lundstrom et al. 1999b). To obtain vectors pSFV($E_{153}$)-GFP, pSFV($E_{324}$), pSFV(T)-GFP, pSFV($TE_{324}$)-GFP, pSFV($PDE_{153}$)-GFP, and pSFV(PDTE)-GFP,, site-directed mutagenesis employing PCR technologies was carried out. Briefly, the SacI-XbaI fragment from pSFV1 (Liljeström and Garoff 1991) was subcloned into the pGEM-7Zf(+) vector (Promega, Madison, Wis., USA). After PCR manipulations, the mutated fragments with the nsP2 mutation Met780Thr and the nsP4 mutations Gly153Glu and Gly324Glu were reintroduced into the pSFV-GFP vector. By using suitable restriction sites, appropriate fragments were replaced in the pSFV(PD)-GFP vector to produce the pSFV($PDE_{153}$)-GFP, pSFV($PDE_{324}$)-GFP, pSFV(PDT)-GFP, and pSFV(PDTE)-GFP constructs. All constructs were verified by sequencing to confirm the accuracy of the mutations. Table 1 summarizes the various vectors and their mutations.

### Generation of SFV stocks

SFV stocks were produced as earlier described (Lundstrom et al. 1994). Briefly, in vitro transcribed RNA molecules from pSFV1-GFP, pSFV(PD)-GFP, pSFV($TE_{324}$)-GFP, pSFV(PDT)-GFP, pSFV($PDE_{153}$)-GFP, pSFV($PDE_{324}$)-GFP, and pSFV(PDTE)-GFP were cotransfected with pSFV-Helper2 RNA into BHK cells. All virus production was performed at 31°C. Virus stocks were harvested at 24 h postelectroporation. SFV stocks were filter-sterilized, activated with α-chymotrypsin (Roche Molecular Biochemicals, Rotkreutz, Switzerland), and the reaction terminated with the trypsin inhibitor aprotinin (Sigma, St. Louis, Mo., USA) prior to use. Approximate virus titers were determined by infection of BHK cells with serial dilutions of virus stocks, followed by fluorescence microscopy examination at 2–3 days postinfection.

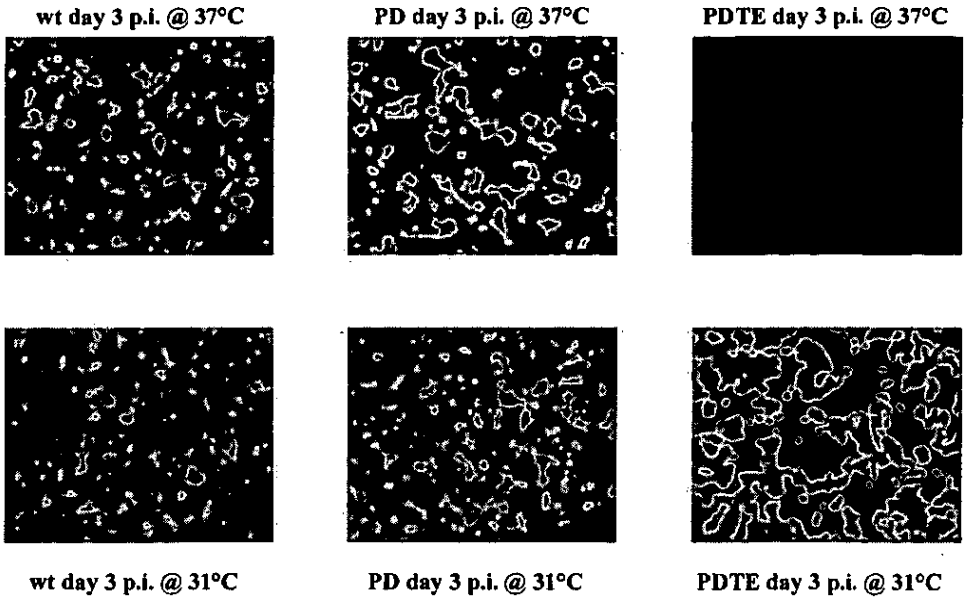
**Table 1** Comparison of relative GFP-fluorescence in baby hamster kidney (BHK) cells infected with wild-type and mutant Semliki Forest virus (SFV) vectors at a multiplicity of infection (MOI) value of 10. Fluorescence measurements were carried out after incubation of BHK cells for 3 days at 31 and 37°C

| Virus | Mutation(s) | 37°C | 31°C |
|---|---|---|---|
| SFV-GFP | Wild-type | 12,086 | 14,188 |
| SFV(PD)-GFP | $S_{259}P$, $R_{650}D$ (nsP2) | 124,850 | 33,769 |
| SFV(T)-GFP | $M_{780}T$ (nsP2) | 7,901 | 33,371 |
| SFV($E_{153}$)-GFP | $G_{153}E$ (nsP4) | 6,749 | 13,876 |
| SFV($E_{324}$)-GFP | $G_{324}E$ (nsP4) | 5,444 | 21,410 |
| SFV($TE_{324}$)-GFP | $M_{780}T$ (nsP2), $G_{324}E$ (nsP4) | 579 | 61,402 |
| SFV(PDT)-GFP | $S_{259}P$, $R_{650}D$, $M_{780}T$ (nsP2) | 27 | 54,443 |
| SFV($PDE_{153}$)-GFP | $S_{259}P$, $R_{650}D$ (nsP2), $G_{153}E$ (nsP4) | 111 | 36,733 |
| SFV($PDE_{324}$)-GFP | $S_{259}P$, $R_{650}D$ (nsP2), $G_{324}E$ (nsP4) | 266 | 58,489 |
| SFV(PDTE)-GFP | $S_{259}P$, $R_{650}D$, $M_{780}T$ (nsP2), $G_{153}E$ (nsP4) | 725[a] | 208,143 |

[a] The seemingly higher value for the SFV(PDTE)-GFP compared to the other temperature-sensitive mutants is a reflection of the relative fluorescence measured for cell cultures. Microscopic examination failed to detect a single GFP-positive cell after infection with SFV(PDTE)-GFP. In contrast, some GFP-positive cells (1 per 5,000 cells) were observed after infection with the mutants SFV($TE_{324}$)-GFP, SFV(PDT)-GFP, SFV($PDE_{153}$)-GFP, and SFV($PDE_{324}$)-GFP and culturing at the non-permissive temperature (37°C).

**Fig. 1A, B** Infection of baby hamster kidney (BHK)-21 cells with wild-type (wt) and mutant Semliki Forest virus (SFV) vectors expressing GFP. **A** Fluorescence micrographs of SFV-infected BHK cells on 6-well plates with an MOI of 10, cultured at 31 and 37°C, and photographed at 3 days postinfection (*p.i.*). **B** Relative fluorescence values as measured directly from the 6-well plates

**A**

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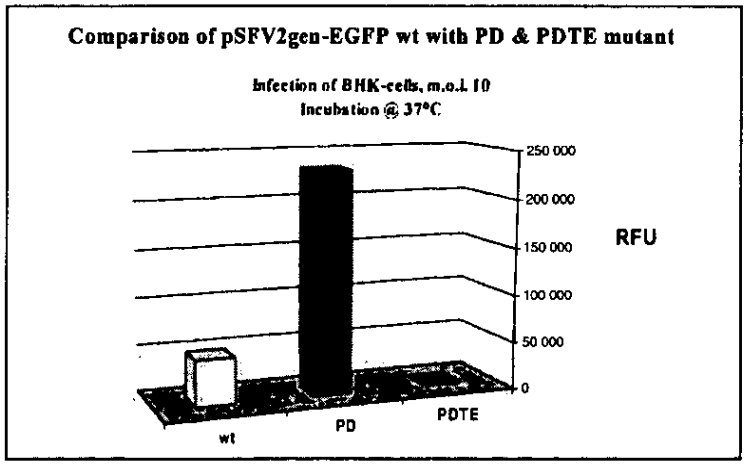
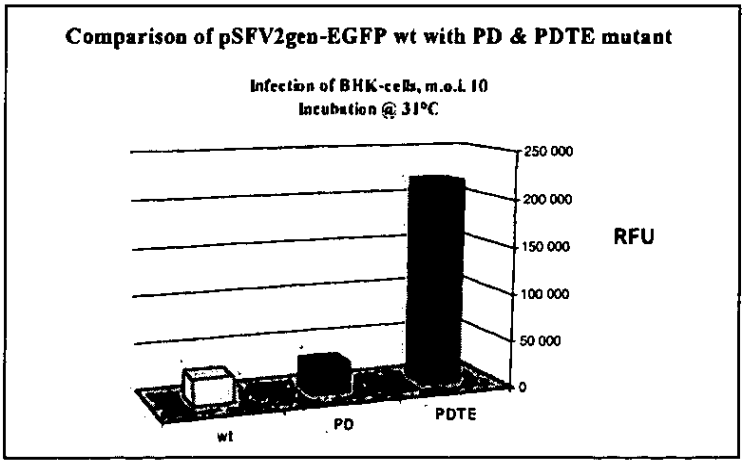


Fluorescence studies in BHK cells and primary neurons

BHK cells and rat hippocampal neurons on 12- or 24-well plates were infected with different multiplicity of infection (MOI) values (1, 5, 10, and 50) of SFV-GFP, SFV(PD)-GFP, SFV($TE_{324}$)-GFP, SFV($PDE_{153}$)-GFP, SFV($PDE_{324}$)-GFP, and SFV(PDTE)-GFP. The GFP expression of infected cells was examined by fluorescence microscopy with a Zeiss Axiovert 100 microscope for at least 7 days. Relative fluorescence intensities were measured in a Fluoroscan Ascent fluorometer (Labsystems, Helsinki, Finland).

Injection of SFV vectors into hippocampal slice cultures

Hippocampal slices from 6 days postnatal rats were prepared according to the roller-tube technique described earlier (Gähwiler 1981). Injection of slice cultures was done as described in detail by Ehrengruber and Lundstrom (2000). Briefly, the slices were transferred to Petri dishes and injection performed with glass micropipettes pulled from borosilicate glass (Clark Electromedical Instruments, Pangbourne, UK). Pipettes containing the viral solutions were lowered into the pyramidal cell layer with the aid of a micromanipulator. Typically 5–15 injection sites were chosen per slice. Upon application of the virus, the slices were washed with culture medium and returned to the roller tubes for further culturing at either 31 or 36°C. Slices were fixed with 4% paraformaldehyde in PBS, and viewed and photographed using a Zeiss Axiovert 100 or Axioplan fluorescence microscope.

## Results

### Mutant SFV vectors

The SFV(PD)-GFP with two point mutations in the nsP2 gene (Ser259Pro and Arg650Asp) has been described earlier (Lundstrom et al. 1999b) leading to a non-cytopathogenic phenotype, similar to the one reported for Sindbis virus (Agapov et al. 1998. Point mutations in the nsP2 and nsP4 genes have been demonstrated to cause a temperature-sensitive phenotype of replication-competent SFV (Hahn et al. 1989). We therefore introduced the nsP2 mutation Met780Thr, and the nsP4 mutations Gly153Glu and Gly324Glu, separately into the SFV-GFP expression vector and could detect the expected temperature-sensitive phenotype with high GFP expression at the permissive temperature (31°C) and strongly reduced fluorescence at the non-permissive temperature (37°C). The combination of the SFV(PD)-GFP vector with the SFV($E_{153}$) mutation, resulting in the triple mutant SFV($PDE_{153}$)-GFP, led to a both non-cytopathogenic and temperature-sensitive phenotype. Interestingly, construction of the quadruple mutant SFV($PDTE_{324}$)-GFP led to an even more pronounced temperature-sensitive vector that showed absolutely no GFP expression in BHK cells at 37°C, even after infection with a relatively high MOI (Fig. 1). Comparison of GFP expression in BHK cells showed that all mutants tested were superior to the wild-type SFV vector. The expression levels were seven- to tenfold higher for SFV(PD) compared to the wild-type SFV-GFP (Fig. 2). Relative fluorescence assays demonstrated that the expression levels were even higher for the triple mutants, SFV($PDE_{153}$)-GFP, SFV($PDE_{324}$)-GFP, and SFV(PDT)-GFP at 31°C (Table 1). Interestingly, the quadruple mutant SFV(PDTE)-GFP showed a further increased expression level (Fig. 3; Table 1). Typically, the onset of the GFP expression was delayed for this mutant, but the expression remained very high for a prolonged time period.

### Expression in hippocampal primary neurons

Infection of rat hippocampal primary neurons in culture revealed a very high infection rate (ca 90%) for SFV-GFP, SFV(PD)-GFP, SFV($PDE_{324}$)-GFP, and SFV(PDTE)-GFP. It was evident that the infection was highly neuron-specific as the primary neurons were cultured on a feeder layer of glial cells which showed hardly any GFP expression (Fig. 4). Similarly, as observed for BHK cells, the expression levels of the SFV mutants SFV(PD)-GFP,

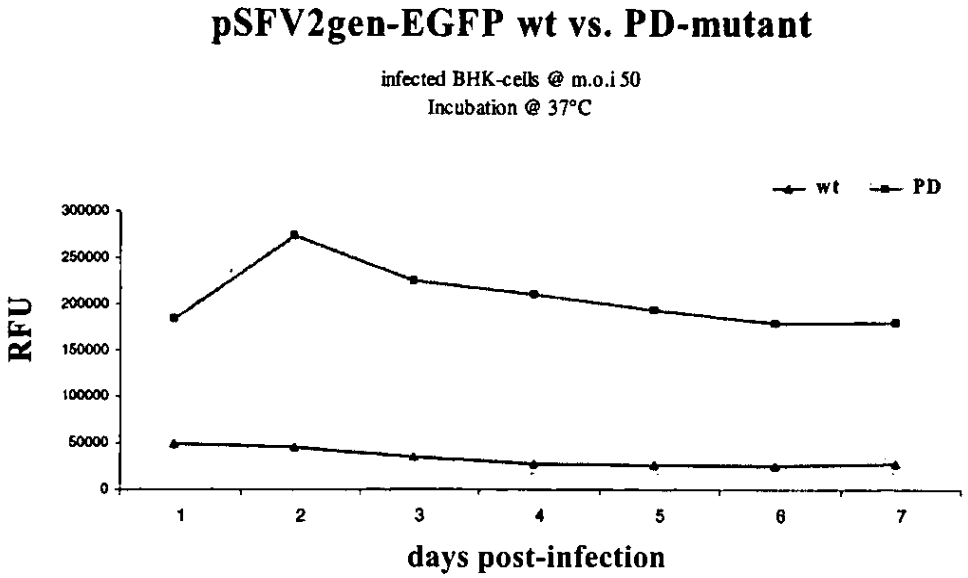
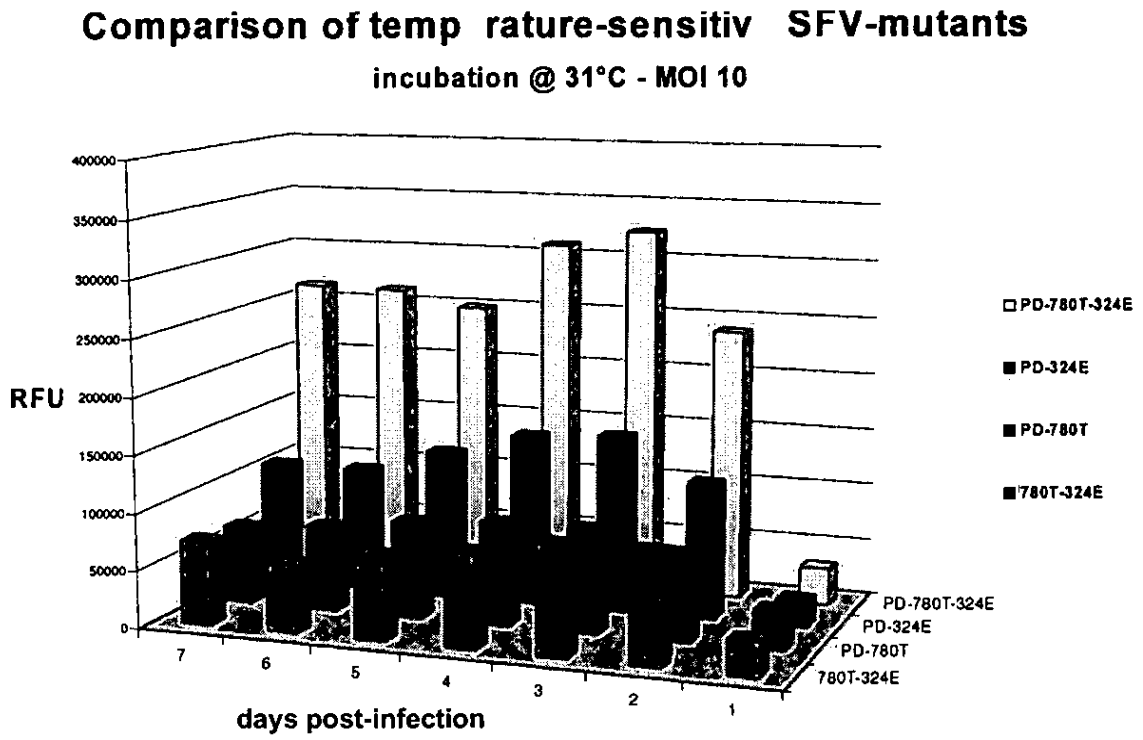



**Fig. 2** Time course of GFP expression from wild-type (wt) SFV-GFP and SFV(PD)-GFP vectors. BHK cells were infected with an MOI of 10 and cultured for 7 days postinfection at 37°C, respectively. Relative fluorescence units (RFU) were measured directly from the cells on 6-well plates



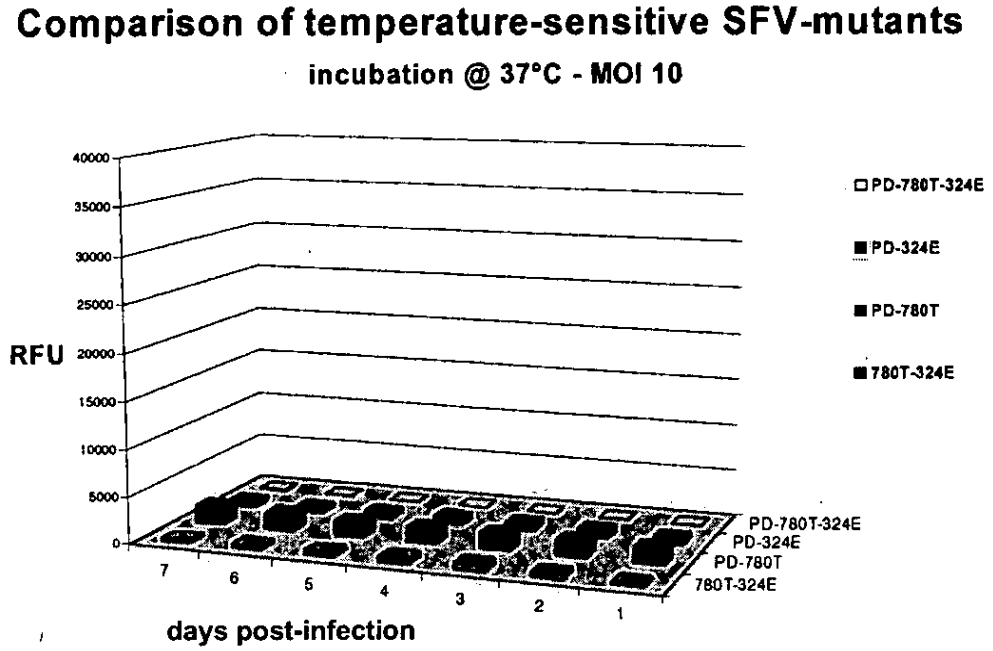




**Fig. 3** Time course of GFP expression from SFV(PDT)-GFP, SFV($PDE_{324}$)-GFP, SFV($TE_{324}$)-GFP, and SFV(PDTE)-GFP vectors. Infected BHK cells were cultured in 6-well plates at 31 and 37°C. Relative fluorescence units (RFU) were measured directly from 6-well plates

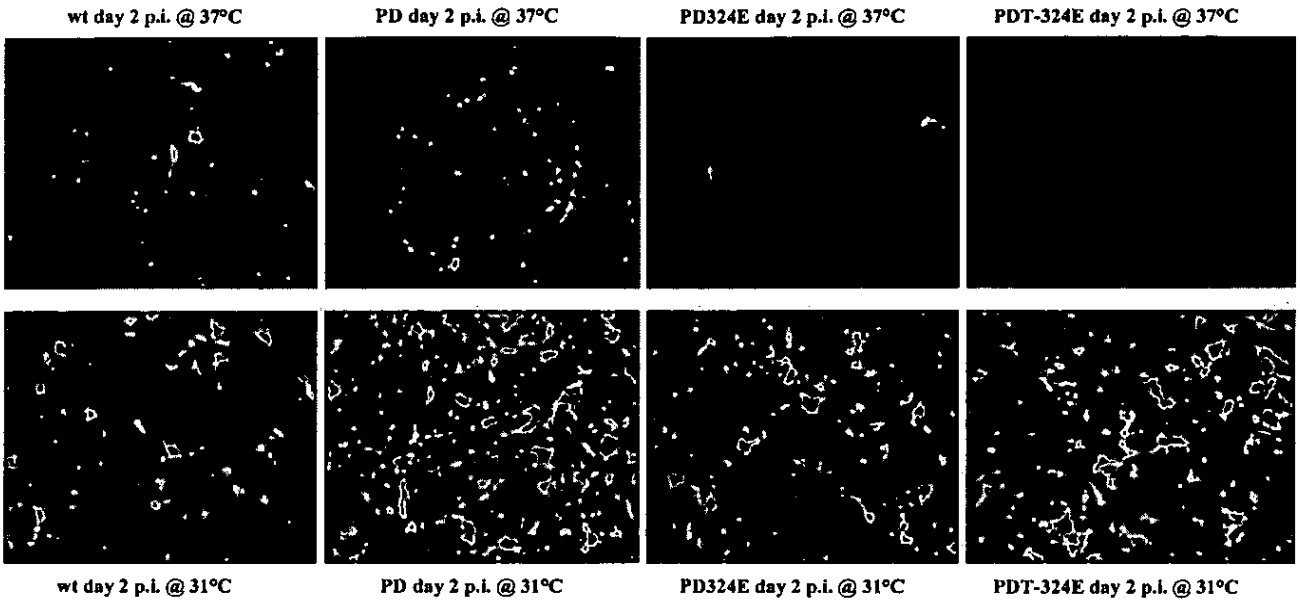



**Fig. 4** Infection of rat primary hippocampal neurons with wild-type and mutant SFV vectors. Fluorescence micrographs of neurons at 2 days postinfection cultured at 31 and 37°C

SFV($PD_{324}$)-GFP, and SFV(PDTE)-GFP were much higher than the ones seen for the wild-type SFV vector. In addition, not a single GFP-positive cell could be detected for the SFV(PDTE) vector when the neurons were cultured at 37°C (Fig. 4). A shift to the permissive temperature (31°C) resulted in high GFP expression in practically every neuronal cell.

Organotypic hippocampal slices

SFV-GFP and SFV(PD)-GFP showed a similar pattern of infection and GFP expression in hippocampal slice cultures. For the same amount of injected virus, there were more GFP-positive cells seen after infection with SFV(PD)-GFP, but for both vectors the preference to infect neuronal cells was very high (>90% of all GFP-positive cells; Ehrengruber et al., manuscript in preparation). Pyramidal cells and interneurons were infected identically by SFV-GFP and SFV(PD)-GFP (ca 85% and ca 6% of all GFP-positive cells, respectively). When slice cultures were injected with SFV($PDE_{153}$)-GFP and cultured at 36°C, by contrast, there was a remarkable, 250-fold decrease of GFP expression in pyramidal cells vs interneurons compared to infections with SFV-GFP (Fig. 5). The number of GFP-positive interneurons, however, was similar to what had been observed for SFV(PD)-GFP. Interestingly, when the temperature was lowered to 31°C the ratio of GFP-expressing pyramidal cells vs interneurons was more similar to the one observed for wild-type SFV-GFP (Fig. 6) and SFV(PD)-GFP (data not shown). For SFV(PDTE)-GFP, similar results were obtained in slice cultures as for BHK cells and primary neurons. No GFP expression was observed at 36°C, whereas at 31°C both pyramidal cells and interneurons were GFP-positive, with a similar ratio between the two neuron types as detected upon infection with wild-type SFV (Fig. 7).

## Discussion

We have demonstrated in this study the usefulness of SFV vectors for gene delivery to neuronal cells. Wild-type SFV vectors give very high infection rates in primary cultures of rat hippocampal neurons (Olkkonen et al. 1993) as well as in hippocampal slice cultures (Ehrengruber et al. 1999). The expression levels of recombinant proteins have been impressive despite the cytotoxicity and the virus-induced shut down of host cell protein synthesis. Thus, electrophysiological recordings have been feasible for SFV-infected neurons. For instance SFV-mediated expression of metabotropic glutamate receptors resulted in functional inhibition of voltage-gated calcium current in primary neurons (Lundstrom et al. 1998). Likewise, electrophysiological recordings were performed from SFV-GFP- and SFV(PD)-GFP-infected CA1 pyramidal cells in hippocampal slice cultures at 1 and 2 days postinjection (Ehrengruber et al., manuscript in preparation).

The development of novel vectors has further increased the application possibilities of SFV. The non-cytopathogenic phenotype expressed by the double mutant vector SFV(PD) should significantly enhance the possibility to use SFV vectors both in vitro and in vivo. Because the host cell protein synthesis is not shut down as dramatically as for the wild-type SFV vector, this vector should be extremely useful and should also allow both antisense and ribozyme technology applications. Furthermore, in hippocampal slice cultures, the triple mutant vector SFV($PDE_{153}$) shows a highly interneuron-selective expression pattern at 36°C. Application of this

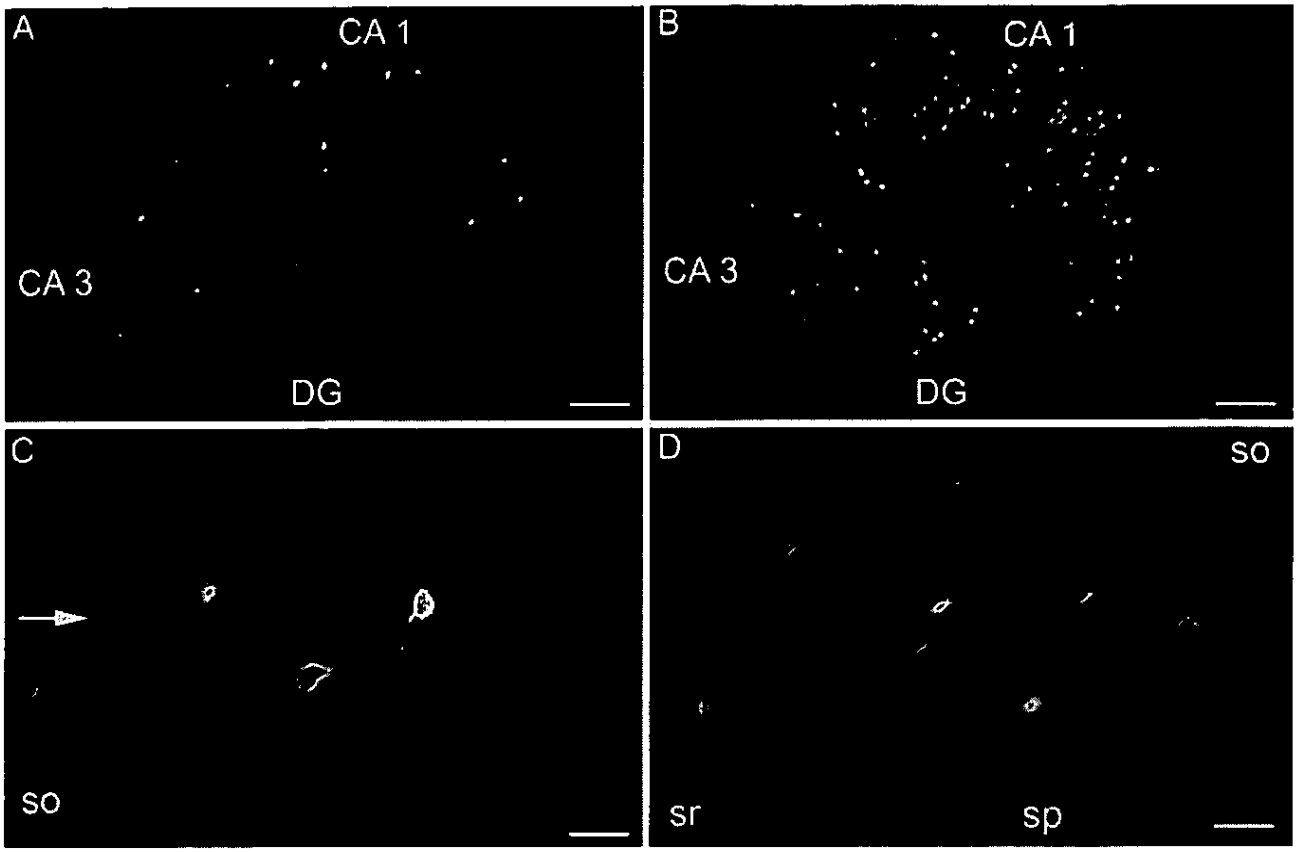



**Fig. 5A–D** Infection of hippocampal slice cultures with SFV ($PDE_{153}$)-GFP. **A, B** Fluorescence illuminations of slices fixed at 17 days in culture and 2 days postinjection. Slices were incubated at 36 (**A**) and 31°C (**B**), respectively. **C** GFP-positive interneuron from CA1 stratum oriens of the slice shown in **A**. The *arrow* indicates the alvear border of the slice. **D** GFP-positive CA1 pyramidal cells of the slice shown in **B**. *DG* Dentate gyrus, *so* stratum oriens, *sp* stratum pyrimidale, *sr* stratum radiatum. *Bars* 270 μm (**A, B**), 65 μm (**C, D**)

SFV mutant thus allows the targeting of transgene expression to interneurons rather than pyramidal cells. Lowering the incubation temperature to 31°C, by contrast, reverts the ratio of GFP-positive pyramidal cells vs interneurons to a ratio more comparable to the ones obtained for wild-type SFV and SFV(PD) vectors. Because the temperature-sensitive mutation in SFV($PDE_{153}$) lies within the nsP4 gene, i.e., the gene encoding the viral RNA polymerase, the mechanism for this interneuron-specific GFP expression resides most likely at the RNA replication level. The SFV($PDE_{153}$) vector, therefore, offers a unique opportunity to study transgene expression specifically in interneurons and can be a useful tool for addressing specific neurobiological topics, such as the control of neuronal network activity by interneuronal activity. Finally, the quadruple mutant SFV(PDTE) exhibits a typical temperature-sensitive phenotype with no GFP expression at the non-permissive temperature, but high transgene expression and prolonged cell survival at the permissive temperature. This vector will be useful in

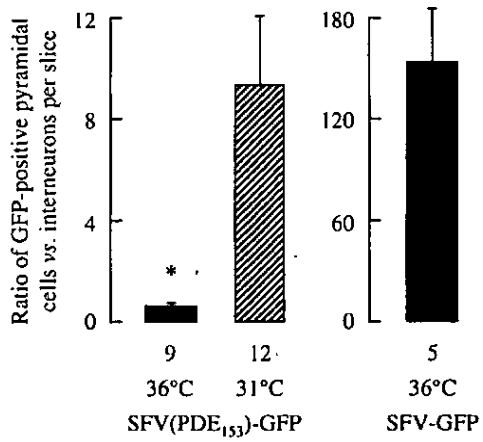



**Fig. 6** SFV($PDE_{153}$) permits the targeting of gene delivery to interneurons. Hippocampal slices prepared from 5- to 6-day-old rats and cultured for 11–25 days were infected with SFV($PDE_{153}$)-GFP (10- to 100-fold dilution; *left*) or with wild-type SFV-GFP (1,000-fold dilution; *right*). Slices were incubated at either 36°C (*black bars*) or at 31°C (*hatched bar*) for 1–5 days. Means ± SEM (*n*) of the ratio between the number of GFP-positive pyramidal cells vs interneurons per slice. Note that the incubation of slices infected with SFV($PDE_{153}$)-GFP at 36°C leads to less GFP-positive pyramidal cells than interneurons (average ratio 0.6) while incubation at 31°C restores the wild-type phenotype with significantly more GFP-expressing pyramidal cells. * Significant difference ($P<0.05$, Student's *t*-test) between this ratio and the corresponding ratio at 31°C

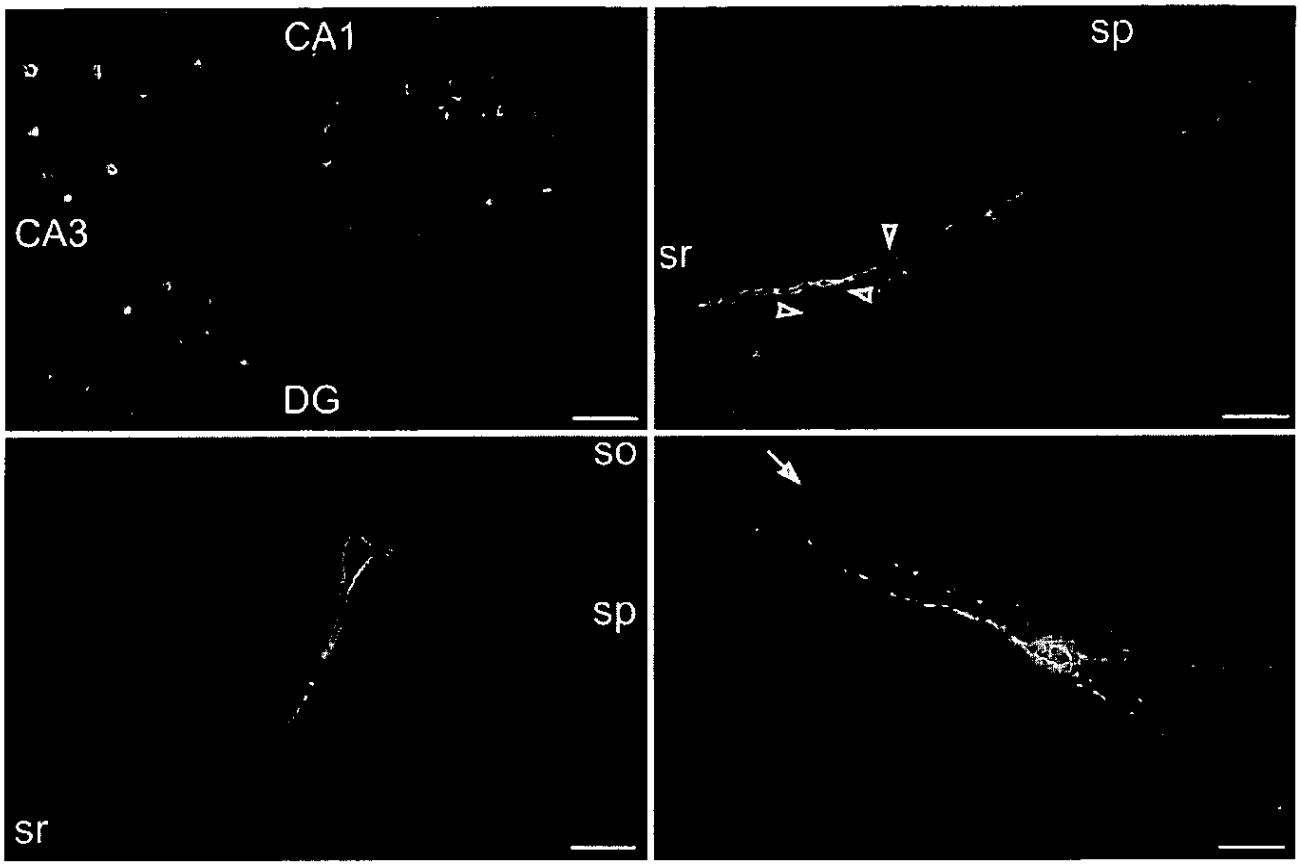



**Fig. 7A–D** Organotypic hippocampal slices infected with SFV (PDTE)-GFP and cultured at 31°C. Fluorescence micrographs of slices fixed at 1 (**C, D**) and 2 (**A, B**) days postinfection. **A** Whole slice. **B** CA1 pyramidal cell; *arrowheads* point to selected GFP-positive dendritic spines. **C** CA3 pyramidal cell. **D** Interneuron in stratum oriens of CA3 region; *arrow* indicates the perimeter of the slice. Abbreviations as in Fig. 5. *Bars* 140 μm (**A**), 15 μm (**B**), 30 μm (**C, D**)

experiments where induction of gene expression should be obtained by simply lowering the incubation temperature of the cell cultures at defined time points postinfection.

In summary, we now have a set of mutant SFV vectors available for in vitro and in situ transfer of genes into neurons. The SFV mutants are characterized by decreased cytotoxicity, increased transgene expression, and temperature-dependent phenotypes, which in cultured hippocampal slices permit the targeting of gene expression to interneurons rather than pyramidal cells.

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# Novel Semliki Forest Virus Vectors with Reduced Cytotoxicity and Temperature Sensitivity for Long-Term Enhancement of Transgene Expression

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**Alphaviral vectors inhibit host cell protein synthesis and are cytotoxic. To overcome these limitations, we modified the nonstructural protein-2 (nsP2) gene in the Semliki Forest virus (SFV) vector, pSFV1. Packaging of SFV replicons with two point mutations in *nsP2* resulted in high-titer recombinant SFV(PD) particles. SFV(PD) led to more efficient host cell protein synthesis, exhibited reduced cytotoxicity and improved cell survival, and allowed greater and prolonged transgene expression than the original vector, SFV. In dissociated hippocampal neurons and organotypic rat hippocampal slices, SFV(PD) infection preserved neuronal morphology and synaptic function more efficiently than SFV. Combination of the two point mutations with a replication-persistent mutation in *nsP2* resulted in a highly temperature-sensitive vector, SFV(PD713P), which efficiently transduced neurons in hippocampal slice cultures. At 31 °C, SFV(PD713P) allowed continuous transgene expression in BHK cells, at amounts comparable to SFV(PD). These new SFV mutants are expected to substantially broaden the application of alphaviral vectors in neurons and other mammalian cells.**

**Key Words: alphavirus, expression vector, metabolic labeling, green fluorescent protein (GFP), hippocampus, neuron, synapse staining, slice culture**

## INTRODUCTION

Recombinant protein expression is an essential part of modern molecular biology research and drug screening processes. Very efficient expression systems have been developed for alphaviruses, mainly Semliki Forest virus (SFV) [1] and Sindbis virus (SIN) [2]. The simple and rapid high-titer virus production and the extremely broad host cell range have permitted efficient transduction of a variety of mammalian cell lines and primary cell cultures [3]. However, a major drawback of alphaviral vectors has been their substantial inhibition of host cell protein synthesis within hours upon infection, resulting in a rapid decrease of endogenous gene expression, induction of apoptosis, and cell death within 72 hours of infection [3,4]. Early cytotoxicity has substantially restricted the applicability of alphaviral vectors and impaired their use for long-lasting experiments, such as kinetic studies or the investigations of specific signal transduction pathways during development.

The alphaviral nonstructural proteins 1–4 (nsP1–nsP4) form the cytoplasmic RNA replicase complex and play essential roles for virus function: nsP1 is required for the initiation of minus-strand RNA synthesis and the capping of viral RNAs, nsP2 contains protease and helicase activity, and nsP4 is the catalytic subunit of the viral RNA polymerase [4]. NsP3 is a phosphoprotein [5] involved in alphaviral RNA replication [6], but its precise function remains unknown. Spontaneous mutations discovered in the nonstructural genes, particularly in nsP2 and nsP4, have considerable effects on the viral pathogenicity.

Mutations in the *nsP2* gene of SIN have produced viruses [7] and replicons [7–9] with greatly reduced cytopathogenicity. The majority of the mutants had a single change at amino acid residue 726. For example, a change at this site from proline to serine produced replicons that had reduced levels of RNA replication and cytopathogenicity [7]; the change from proline to leucine led to a further reduction both in RNA synthesis and cytopathogenicity [8]. Temperature-sensitive mutations have been



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described for SIN in each of the *nsP* genes [10,11], and those in *nsP4* [12] have been introduced into SIN expression vectors. Combination of the P726S change in *nsP2* and a temperature-sensitive mutation in *nsP4* led to the inducible expression of replicons in a variety of cell lines [12].

A study on replication-competent SFV also revealed a point mutation (R649D) in the *nsP2* nuclear localization signal to confer a lower virulence in mice [13,14]. Furthermore, the introduction of known temperature-sensitive mutations into *nsP2* and *nsP4* reduced the cytotoxicity of SFV vectors [15]. In addition, random mutagenesis of *nsP* genes in SIN and SFV vectors containing the neomycin resistance gene generated replication-persistent vectors that had either deletions or point mutations in *nsP2* [9].

Here we describe (1) a vector based on the SFV4 strain, SFV(PD), harboring two point mutations in *nsP2* (S259P, R650D), as well as (2) a temperature-sensitive vector, SFV(PD713P), wherein the replication-persistent L713P mutation in *nsP2* was introduced into SFV(PD). These novel SFV vectors were found to be less cytotoxic than the original SFV vectors, and lead to higher transgene expression levels in mammalian cell lines, primary hippocampal and cortical neurons, and organotypic hippocampal slices. In addition, host cell protein synthesis, neuronal morphology and dendritic arborization, synapses, and synaptic vesicular exo- and endocytosis in infected cells were much better conserved than with original SFV vectors.

## Results

### Effect on Host Cell Protein Synthesis

In contrast to the strong inhibition of endogenous gene expression by the SFV4-based vector, infection with SFV(PD) encoding LacZ, luciferase, and hNK1R still permitted substantial host cell protein synthesis at 16 hours post-infection in BHK and HEK 293 cells, as determined by metabolic labeling (Figs. 1A and 1B, respectively). Similar results were found in Chinese hamster ovary cells (data not shown). Likewise, in rat E18 hippocampal neurons, wherein infection with SFV-LacZ produced a substantial inhibition of host cell protein expression with high levels of β-galactosidase, infection with SFV(PD)-LacZ permitted a substantial amount of host protein synthesis as compared to uninfected neurons (Fig. 1C). These metabolic labeling experiments were conducted on cell populations; it is thus possible that infection with SFV(PD) had inhibited host protein synthesis in some cells more than in other cells, rather than equally in all cells. In any case, our data indicate that SFV(PD) has a substantially reduced impact on the endogenous protein synthesis of cell lines and neuronal cells.

### Transgene Expression Levels and Survival of Infected Cells

SFV vectors with the single point mutations S259P, R649D, R650D, and L713P in *nsP2* expressed slightly increased levels of recombinant protein when compared to the original SFV vector (data not shown). However, when we combined the S259P and R650D mutations into SFV(PD), we found a synergistic effect that resulted in a substantial increase in transgene expression. Compared to SFV-GFP, the onset of transgene expression obtained with SFV(PD)-GFP at up to 1 day post-infection was sometimes delayed (for example, Fig. 2C), but the maximal GFP fluorescence levels achieved at 2–3 days post-infection in BHK cells (Figs. 2A, 2B) and rat E18 cortical neurons (Fig. 2C) were ~ 6- and ~ 2-fold higher, respectively. A similar effect was found for the LacZ reporter gene in BHK cells, where SFV(PD)-LacZ caused a 6-fold larger expression than SFV-LacZ (300 ± 2 vs. 50 ± 1 ng β-galactosidase/μl cell lysate, respectively) (Fig. 2D). These results show that the decreased impact of SFV(PD) on host cell protein synthesis (see earlier) is paralleled by increased transgene expression.

SFV and SFV(PD) exhibited substantial differences in their effect on host cell survival. Microscopic examination of BHK and HEK 293 cells revealed that at 37 °C SFV(PD)-GFP caused less cytopathic effect (CPE, rounding up and detachment of cells) than SFV-GFP (Fig. 3A, right, and data not shown). Whereas most SFV-GFP-infected cells showed CPE within 48 hours, the majority of SFV(PD)-GFP-infected cells appeared intact for at least 5 days. In agreement with this observation, the time course of GFP as well as β-galactosidase expression in BHK cells infected with SFV(PD) was longer than for cells infected with SFV (10 vs. ~ 6 days, respectively; Figs. 2A, 2D). A similar prolonged expression was found in rat E18 cortical neurons (Fig. 2C). Only when SFV(PD) was used at higher concentration (multiplicity of infection (MOI) > 5 and > 1 for BHK cells and P4–5 hippocampal neurons, respectively), cytotoxicity was observed (data not shown). In both BHK cells and neurons, the decrease in GFP fluorescence found after 3 days for wild-type SFV and 7 days for SFV(PD) was mostly due to the lysis of infected cells as well as, presumably, the possible antiviral agents, such as interferon, induced under conditions in which host cell protein synthesis is not inhibited. In any case, our results demonstrate that survival of infected BHK and HEK 293 cells as well as primary neurons is longer for SFV(PD) than for wild-type SFV. This extended cellular survival presumably relates to SFV(PD) still permitting host cell protein synthesis (compare with earlier discussion).

The L713P mutation in nsP2 has earlier been described to permit persistent SFV RNA replication in BHK cells [9]. We thus introduced it into SFV(PD) to generate the triple mutant SFV(PD713P). Interestingly, this vector had a temperature-sensitive phenotype with very low transgene expression at 37 °C (Figs. 2B and 3A, right). However, when

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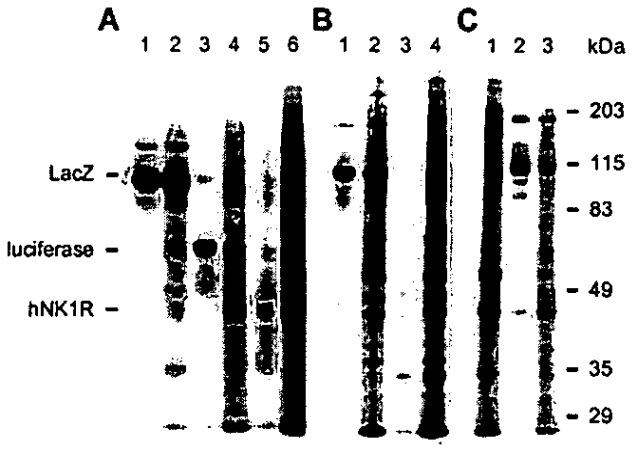


FIG. 1. Metabolic labeling of BHK and HEK 293 cells as well as rat hippocampal neurons infected with conventional SFV and SFV(PD). Expression was verified by 10% SDS-PAGE. Autoradiograms of cells incubated at 37 °C and labeled with [$^{35}$S]methionine at 16 hours post-infection. (A) BHK cells infected with SFV-LacZ (lane 1), SFV(PD)-LacZ (lane 2), SFV-luciferase (lane 3), SFV(PD)-luciferase (lane 4), SFV-hNK1R (lane 5), and SFV(PD)-hNK1R (lane 6). (B) HEK 293 cells infected with SFV-LacZ (lane 1), SFV(PD)-LacZ (lane 2), SFV-hNK1R (lane 3), and SFV(PD)-hNK1R (lane 4). (C) Uninfected rat E18 hippocampal neurons (lane 1) and neurons infected with SFV-LacZ (lane 2) and SFV(PD)-LacZ (lane 3). Note the dark "smears" of radioactively labeled host cell proteins in uninfected neurons and cells infected with SFV(PD), which are absent in cells infected with wild-type SFV. The bands for the expressed transgenes are marked on the left and the molecular weights of the marker proteins are indicated on the right.

SFV(PD713P)-GFP-infected BHK cells were cultured at 31 °C, the GFP expression levels obtained at ≥ 3 days post-infection were similar or even higher than those obtained with SFV(PD)-GFP (Figs. 2A and 3A, left). At this temperature, the maximal GFP expression levels obtained with SFV(PD713P) were doubled as compared to the ones yielded with SFV(713P) carrying the single L713P mutation (Fig. 2B). This result shows that SFV(PD713P) provides stronger transgene expression than the original SFV(713P) mutant described by Perri *et al.* [9].

Most importantly, whereas SFV(PD)-mediated GFP expression declined with time, SFV(PD713P) allowed it to proceed at elevated levels for 20 days (that is, the longest period tested; Fig. 2A). In accordance with this finding, SFV(PD713P)-infected cells showed hardly any CPE and could even undergo mitosis and be recultured (data not shown). These results agree with the earlier described persistence of the L713P mutant SFV [9] and suggest that SFV(PD713P)-encoded viral replication continues for an extended period. Considering all these results, SFV(PD713P) allows both high-level and long-term transgene expression.

### Temperature Effects

We have demonstrated earlier that the temperature used for culturing SFV-infected cells can substantially affect

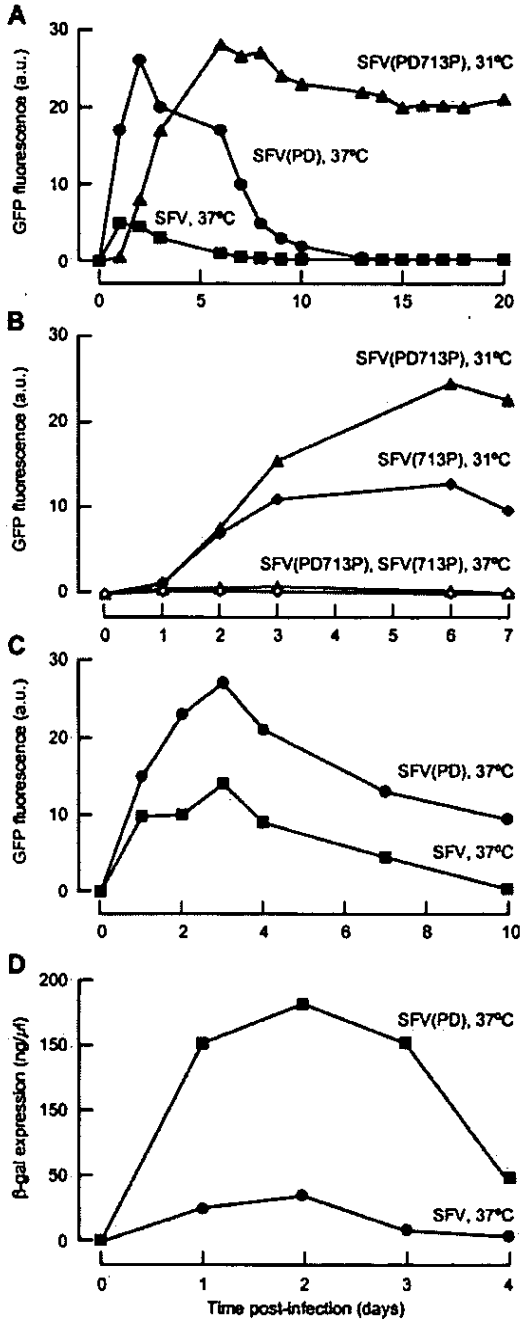


FIG. 2. Time course of transgene expression in BHK cells (A, B, D) and rat E18 cortical neurons (C). Cells were infected with wild-type SFV-GFP (squares), SFV(PD)-GFP (circles), SFV(PD713P)-GFP (triangles), SFV(713P)-GFP (open diamonds), (D) SFV-LacZ (circles) and SFV(PD)-LacZ (squares). Relative fluorescence was measured in living cells at increasing time points post-infection. Cells infected with SFV-GFP and SFV(PD)-GFP were cultured at 37 °C, whereas cells infected with SFV(PD713P)-GFP and SFV(713P)-GFP were cultured at both 37 °C and 31 °C (as indicated). Note that the maximal fluorescence levels for SFV(PD) are similar to the ones for SFV(PD713P) (A), which in turn are higher than for SFV(713P) (B). β-Galactosidase activity was measured in homogenates from parallel cultures at various time points post-infection (D). a.u., Arbitrary units.

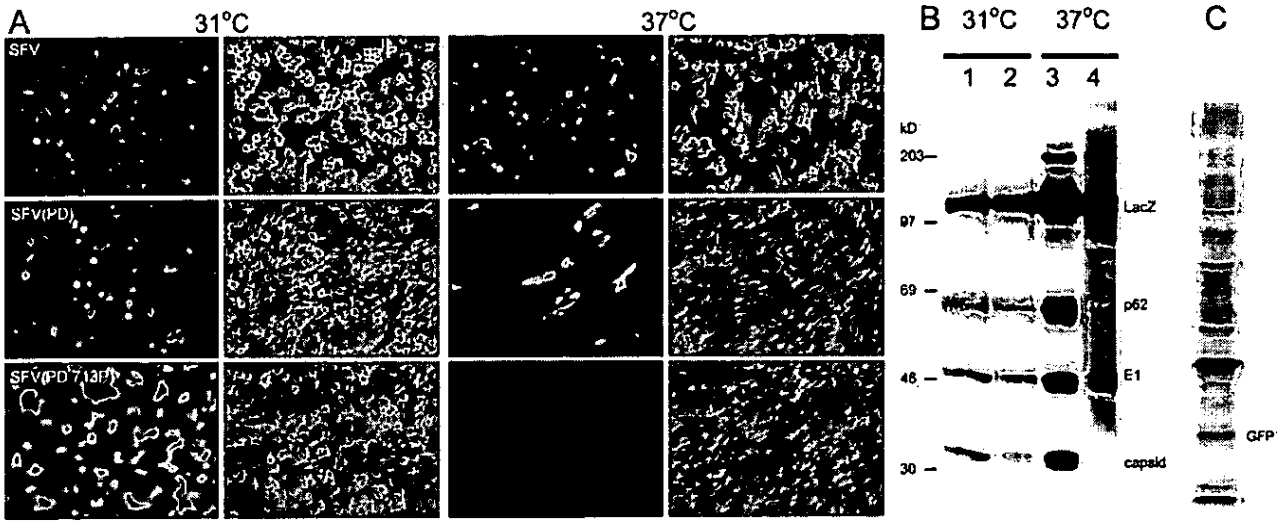


FIG. 3. Temperature effect on GFP expression and morphology (A) as well as protein synthesis (B, C) in BHK cells. (A) Fluorescence and phase contrast micrographs of cells at 3 days after infection with SFV-GFP (top), SFV(PD)-GFP (middle), and SFV(PD713P)-GFP (bottom), and culturing at 31 °C and 37 °C (left and right, respectively). (B) Metabolic labeling of cells co-electroporated with RNA from pSFV-Helper2 and wild-type pSFV3-LacZ (lanes 1 and 3) or pSFV(PD)-LacZ (lanes 2 and 4). Cells were incubated at 31 °C and 37 °C (lanes 1–2 and 3–4, respectively). Expression was verified by 10% SDS-PAGE followed by autoradiography. Note the absence of labeled host cell proteins in lane 2. The bands for the expressed viral proteins (LacZ reporter, p62 precursor for spike proteins E3 and E2, spike protein E1, and capsid protein) and the molecular weights of marker proteins are indicated. (C) Metabolic labeling of BHK cells (31 °C) infected with SFV(PD713P)-GFP for 16 hours and verified by SDS-PAGE and autoradiography.

transgene expression levels [16]. In addition, many mutations in *nsP1–nsP4* of alphaviruses cause temperature-sensitive phenotypes [10–12]. We therefore investigated for SFV(PD) and SFV(PD713P) the effect of altered temperature on virus production, transgene expression, and host cell protein synthesis in BHK cells.

The titers of SFV(PD) replicons obtained at 31 °C were 5- to 10-fold higher than at 37 °C. In contrast, this temperature change had no substantial effect on the production of the SFV4-based replicons. The production of SFV(PD713P) replicons was highly temperature-dependent, again. Extremely low titers resulted at 37 °C ($< 10^3$ infectious particles/ml), whereas higher titers occurred at 31 °C ($10^7$ infectious particles/ml). Table 1 summarizes the titers obtained for the different viral constructs at 31 °C and 37 °C.

Whereas changing the temperature from 37 °C to 31 °C did not substantially alter the GFP fluorescence levels obtained with conventional SFV, the levels for SFV(PD) at 37 °C were at least 5-fold higher at 31 °C, when they were comparable to the ones for conventional SFV (Fig. 3A): the mean fluorescence at 37 °C was 49,800 and 272,800 arbitrary units (a.u.) for SFV-GFP and SFV(PD)-GFP, respectively. SFV(PD713P)-mediated GFP expression showed an even more pronounced temperature sensitivity, in that no detectable levels occurred at 37 °C (Fig. 3A, right), but at 31 °C they were similar to or higher than the ones for SFV(PD) (Figs. 2A and 3A, left). Table 1 gives an overview of the transgene expression levels obtained at 31 °C versus 37 °C.

The effect of SFV(PD) on host cell protein synthesis strongly depended on the incubation temperature. Similar to cells infected with SFV(PD) (Fig. 1), host cell protein synthesis continued to occur in cells electroporated with SFV(PD) RNA (Fig. 3B, lane 4). However, when SFV(PD) RNA-transfected cells were cultured at 31 °C, host cell

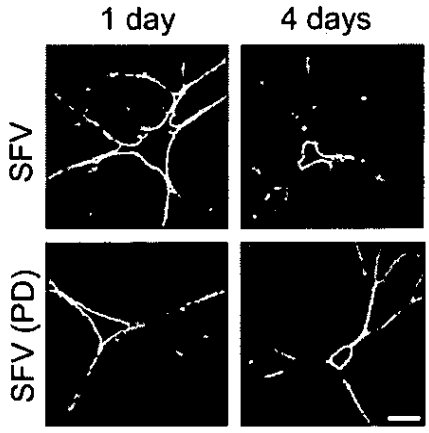


FIG. 4. Infection of dissociated rat hippocampal neurons. Dissociated rat hippocampal neurons prepared from P4–5 rats were cultured for 2 weeks and then infected with wild-type SFV-GFP (top) and SFV(PD)-GFP (bottom). Fluorescence micrographs show the GFP fluorescence (green) and synaptic vesicle cycling (red) of neurons at 1 and 4 days post-infection (left and right, respectively). Note the abnormal dendritic tree and the reduced number of active synapses in neurons infected with wild-type SFV, both very consistent findings at 4 days post-infection. Bar represents 100 μm.

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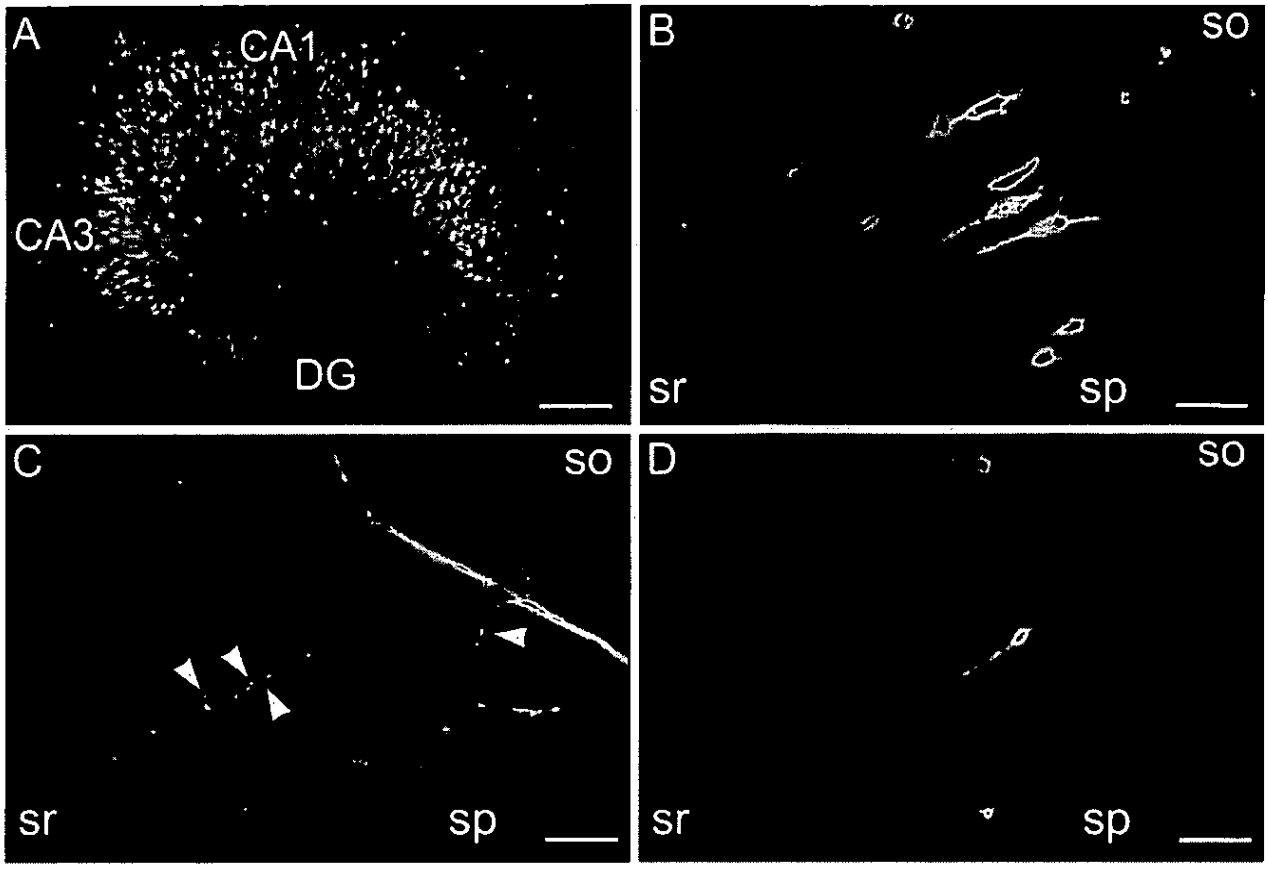


FIG. 5. Infection of cultured rat hippocampal slices. Fluorescence illuminations of slices at 2 and 3 days after injection of SFV(PD)-GFP (A–C) and SFV(PD713P)-GFP (D), respectively, into stratum pyramidale. Upon infection, slices were cultured at 37 °C (A–C) and 31 °C (D). (A) Whole slice; (B, D) CA3 and CA1 pyramidal cells, respectively; (C) apical dendrites in CA1 stratum radiatum with arrowheads pointing to selected $GFP^+$ spines. DG, dentate gyrus; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum. Bars represent 320 μm (A), 80 μm (B, D), and 25 μm (C).

protein synthesis was efficiently turned off (Fig. 3B, lane 2), as was the case for cells transfected with RNA for SFV (Fig. 3B, lanes 1 and 3), and mainly synthesis of virus-encoded proteins occurred. In agreement with the block of host cell protein synthesis at 31 °C, cells infected with SFV(PD) displayed massive CPE at 31 °C in a manner similar to conventional SFV (Fig. 3A, left). Interestingly, as described earlier, infection with SFV(PD713P) at 31 °C did not cause any CPE but rather permitted continuous cellular survival and transgene expression (compare Fig. 2A). In any case, our data show that SFV(PD) exhibits a less cytopathic phenotype at 37 °C but not 31 °C. Again, Table 1 summarizes the effect of the incubation temperature on host cell protein synthesis and survival after infection with the different viral constructs.

### Survival and Synaptic Activity of P4–5 Hippocampal Neurons

To avoid overinfection of neurons (see earlier), we used SFV-GFP and SFV(PD)-GFP at 100 and 1,000 particles/ml, respectively. At these concentrations, very few neurons per dish were infected. They were imaged 1 and 4 days post-infection. Whereas infection with SFV induced abnormal neuronal morphologies in the form of swellings of dendritic processes followed by the complete disorganization of dendrites and CPE (Fig. 4, top right panel), SFV(PD)-infected neurons remained morphologically intact (Fig. 4, bottom right panel). As a measure of neuronal function, we examined presynaptic quantal release by monitoring vesicular uptake of extracellular tracers. In these experiments, evoked quantal release was triggered by electrical stimulation at 0.5 Hz in the presence of anti-synaptotagmin-I antibodies. These antibodies specifically recognize the luminal domain of the synaptic vesicle protein and, upon exocytotic fusion, bind to exposed synaptotagmin-I and are then taken up into the presynaptic terminal by vesicle recycling [17]. Our quantitative analysis of the immunoreactivity in axon terminals at both 1 and 4 days post-infection showed that SFV(PD)-

**Table 1:** Comparison of the conventional and novel SFV vectors as a function of incubation temperature

| | Titers obtained (particles/ml) | | Transgene expression | | Host protein synthesis | | Induction of CPE | |
|---|---|---|---|---|---|---|---|---|
| Vector | 31 °C | 37 °C | 31 °C | 37 °C | 31 °C | 37 °C | 31 °C | 37 °C |
| SFV | $\sim 10^9$ | $\sim 10^9$ | + | + | − | − | + | + |
| SFV (PD) | $0.5–1 \times 10^8$ | $0.5–2 \times 10^7$ | + | ++ | − | + | + | − |
| SFV (PD713P) | $\sim 10^7$ | $<10^3$ | + | − | + | n.a. | − | n.d. |

The table summarizes the viral titers obtained at 31 °C and 37 °C and schematically summarizes transgene expression, effect on host cell protein synthesis, and induction of cytopathic effects (CPE) of the vectors at both temperatures. Note that the incubation temperature drastically alters the behavior of SFV(PD) and SFV(PD713P). −, No detectable expression; +, relatively high expression; ++, 2- to 5-fold higher expression; n.d., not determined; n.a., not applicable.

infected neurons not only retain a normal morphology but also display normal synapses with detectable levels of evoked synaptic exo- and endocytosis. On the contrary, wild-type SFV-infected cells displayed very few active synapses at 4 days post-infection (Fig. 4, top right panel). Taken together, our data demonstrate that SFV(PD), as compared to SFV, maintains unaltered synaptic physiology and permits substantially longer survival of hippocampal neurons.

### Infection of Hippocampal Slices

We have demonstrated earlier high neuronal specificity of SFV-mediated transgene expression by using SFV vectors in rat hippocampal slices [18]. Here, we injected SFV(PD)-GFP at 10- to 1000-fold dilution, corresponding to $\sim 10^5$ to $10^3$ infectious particles, into hippocampal slices and then incubated them at 37 °C. GFP fluorescence could be detected at 1 day post-infection and was increased at 2 days, when it allowed the unambiguous identification of infected CA1 and CA3 pyramidal cells (Figs. 5A, 5B). At this and subsequent time points, GFP fluorescence could easily be detected in very distal and thin dendrites of up to 4th order and even in presumptive spines (Fig. 5C). $GFP^+$ neurons appeared morphologically intact for at least 4 days post-infection. Upon application of 200 SFV(PD)-GFP particles, an average of 105 cells in the injected area per slice expressed GFP fluorescence at 1–2 days post-infection; ~ 87% of them were pyramidal cells and ~ 5% interneurons [19]. Overall, these findings are similar to the results earlier obtained with SFV-GFP [18] and show that SFV(PD) also efficiently transduces neurons in rat hippocampal slices.

We also applied SFV(PD713P)-GFP to hippocampal slices. Because the titers obtained for SFV(PD713P)-GFP were substantially lower than for wild-type SFV and SFV(PD), we injected undiluted SFV(PD713P)-GFP and a 10-fold dilution. Upon incubation at 31 °C, only single $GFP^+$ pyramidal cells were found for the 10-fold dilution ($n$ = 12 slices). The undiluted virus, by contrast, resulted in an average of 20 ± 5 $GFP^+$ cells per slice ($n$ = 5 slices). Similar to BHK cells, no GFP expression was detected when the slices were cultured at 37 °C. Figure 5D shows a SFV(PD713P)-GFP-infected CA1 pyramidal cell at 3 days post-infection. As a result of the high expression levels obtained at 31 °C, GFP fluorescence could be detected in extremely distant and thin dendrites. Again, these data are compatible with the efficient transduction of neurons in tissue slices that we earlier obtained with wild-type SFV and SFV(PD) [18,19].

## Discussion

We have engineered two novel SFV vectors, SFV(PD) and SFV(PD713P), that generate infectious recombinant particles and, upon infection of rodent and human cell lines as well as neurons, bring about 2- to 6-fold elevated levels of expressed recombinant protein, as compared to the wild-type SFV vector. A similar increase in transgene expression has been earlier described in SIN for the S726P mutation in *nsP2* (P726S) [7]. In addition, both SFV(PD) and SFV(PD713P) led to substantially prolonged host cell survival and transgene expression, with SFV(PD713P) causing persistent GFP expression. The decrease of cytotoxicity obviously results from the ability of these replicons—in contrast to conventional SFV—to permit the synthesis of host cell proteins, which are required for infected cells to survive. Such a reduced inhibition of endogenous protein synthesis also underlies the persistent infection obtained in BHK cells with SIN carrying the P726S mutation in *nsP2* [7]. Because uninfected cells synthesize more total protein than alphavirus-infected cells [4], we assume that these SFV and SIN mutants cause elevated transgene expression by providing infected cells with a higher ability for total protein synthesis.

Because nsP2 is the protease responsible for cleaving the nsP1–nsP4 polyprotein, the L713P mutation might result in altered proteolytic activity. However, no such alteration was found for the corresponding mutation in *nsP2* from SIN [7]. In SFV-infected cells, about half of the nsP2, which also contains NTPase activity [20], is transported to the nucleus [21]. A point mutation (R649D) in the nuclear localization signal ($P^{648}$RRV) of nsP2 made it cytoplasmic [22] and, when incorporated into SFV particles, reduced the damage of host cell DNA synthesis and

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the pathogenicity in mice [13]. Because the R650D mutation in SFV(PD) also lies within the nuclear localization signal, we suggest that the nsP2 of SFV(PD) remains cytosolic in host cells. In support of this hypothesis, our preliminary data indicate that this is the case, at least for BHK cells, in which no nsP2 was detected in the nucleus, but was retained in the cytoplasm based on immunofluorescence microscopy using nsP antibodies (M. J. Fend and M. U. Ehrengruber, unpublished results).

Importantly, when SFV(PD) was used at higher MOI, it was cytotoxic to both BHK cells and hippocampal neurons in a manner similar to SFV. The reason may be that the extreme amounts of viral RNA molecules generated by multiple viral genomes in a given cell efficiently compete with endogenous RNA molecules for the cellular translation machinery. In addition, it has been shown that double-strand RNA molecules generated during alphaviral replication induce interferon, which can upregulate cytotoxic protein kinase-RNA and 2′,5′-oligoadenylate [23]. However, our preliminary DNA microarray analysis using HEK 293 cells did not identify any interferon gene to be induced by SFV(PD) (U. Certa and K. Lundstrom, unpublished results). In any case, experiments aiming at 100% infection levels with SFV(PD) should be designed carefully to prevent overinfection and, thus, cytotoxicity. It is therefore highly advisable to conduct titration experiments for various host cells to find out the limits for cytotoxicity. For instance, in BHK cells and neurons, MOI values $< 5$ and $< 1$, respectively, are suggested.

It is interesting that SFV(PD) is less cytotoxic at 37 °C but not at 31 °C. The two mutations in nsP2 might affect viral RNA replication at 37 °C rather than at 31 °C. In support of this idea, the titer of SFV(PD) stocks generated at 37 °C was substantially lower than at 31 °C. Interestingly, SFV(PD713) behaved quite differently than SFV(PD). It was strongly temperature sensitive and had to be packaged at the permissive temperature (31 °C), but it was not cytotoxic at this temperature.

In summary, our novel, less cytopathic SFV mutants will allow application of SFV vectors under conditions at which the cell functions are not compromised by endogenous gene inhibition and early onset of apoptosis. In addition to general overexpression of recombinant proteins, the SFV technology can now be used more efficiently to study expression kinetics and signal transduction events. Additionally, the absence of the general inhibition of endogenous gene expression should further increase the application range of the SFV mutants. For instance, antisense and RNA interference approaches should be especially attractive because of the extreme cytoplasmic RNA amplification from the SFV replicon. Our preliminary results demonstrated that co-infection with SFV(PD) carrying sense and antisense target sequences efficiently and specifically reduced the target gene expression in various host cell lines (U. Certa and K. Lundstrom, unpublished results). In the area of gene therapy, SFV(PD) has two advantages over the SFV4-based vector: (1) the higher expression levels results in production of more therapeutic protein, and (2), the prolonged survival of host cells allows for improved treatment efficacy. As compared to the earlier described less cytopathic and noncytopathic SIN vectors [7,8,24], the advantages of SFV(PD) and SFV(PD713P) are the wider host cell range (cell lines and neurons) and, at least for SFV(PD), the higher replicon titers obtained.

## MATERIALS AND METHODS

***Cell and tissue cultures.*** BHK, Chinese hamster ovary, and HEK 293 cells were cultured in a 1:1 mixture of Dulbecco's modified F-12 medium (Invitrogen) and Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 4 mM glutamate and 10% FCS. Primary embryonic day 18 (E18) rat hippocampal and cortical neurons were cultured in Neurobasal medium (Invitrogen) on 24-well plates, while postnatal day 4–5 (P4–5) rat hippocampal neurons were cultured on glass coverslips in 35-mm petri dishes as described [17]. Briefly, the CA1 and CA3 hippocampal regions were removed from 3- to 5-day-old rats, and the neurons recovered by enzymatic digestion (trypsin type XI, 10 mg/ml, plus DNase I type IV, 0.5 mg/ml) and mechanical dissociation. The cells were cultured in minimal essential medium containing 0.6% (wt/vol) glucose, 1 mM glutamine, 2.4 g/L $NaHCO_3$, 100 mg/ml bovine transferrin, 25 mg/ml insulin, 5–10% FCS, and plated at a density of 50,000 cells per 35-mm plastic petri dish (Falcon) coated with polyornithine and Matrigel (Collaborative Research). The cultures were maintained at 37 °C in 95% air, 5% $CO_2$ in a humidified incubator, and the medium replaced every 3–4 days. From the second day in culture, the culture medium was supplemented with 5 μM cytosine-D-arabinofuranoside. Neurons were used for synaptic uptake experiments at 10–14 days after plating. Hippocampal slices from postnatal day 6 rats were cultured in the roller-tube configuration [25].

***Site-directed mutagenesis.*** Amino acid substitutions in the protein encoded by the SFV *nsP2* gene were carried out by PCR technology. The nsP2–nsP4 region was subcloned as a *SacI-XbaI* fragment from pSFV2gen [26] into pGEM7Zf(+) (Promega). pGEM7Zf(+)-nsP2-4 was then used as a template for PCR reactions with primers designed to substitute the desired nucleotides in the *nsP2* gene. The mutations were confirmed by sequencing, and the entire *SacI-XbaI* fragment reintroduced into pSFV2gen.

***Generation of recombinant SFV.*** RNA from *in vitro*-transcribed pSFV3 encoding *Escherichia coli* β-galactosidase (LacZ) [1], pSFV1 encoding firefly luciferase [27] and human neurokinin 1 receptor (hNK1R) [28], pSFV2gen encoding enhanced green fluorescent protein (GFP) [18], pSFV(PD)-LacZ, pSFV(PD)-luciferase, pSFV(PD)-GFP, pSFV(PD)-hNK1R, pSFV(713P)-GFP, and pSFV(PD713P)-GFP was co-electroporated with RNA from pSFV-Helper2 [29] into BHK cells. Recombinant SFV particles were harvested after 24 hours and activated with α-chymotrypsin, and test infections with serial dilutions of virus expressing GFP and LacZ were carried out for titer determinations as described [30].

***Infection and analysis of gene expression.*** Cell lines and primary neurons were infected by adding undiluted (cell lines) or diluted (neurons) virus to the culture medium. Organotypic hippocampal slices (average age: 15 ± 6 days in culture, mean ± SD, 7 batches; 2–14 sister cultures per batch) were infected and analyzed as described [18,31]. Upon infection, cells and slices were incubated at either 31 °C or 37 °C (as indicated).

Cells were metabolically labeled at 16 hours post-infection with [$^{35}$S]methionine (Perkin Elmer, Zurich, Switzerland), and protein expression verified by 10% SDS-PAGE and subsequent autoradiography. LacZ expression was visualized by X-gal staining, and enzymatic activity measured as described [1]. GFP fluorescence was monitored in living cells with a Zeiss Axiovert 100 microscope and levels quantified on 24- and 96-well plates by using a Fluoroscan Ascent fluorometer (Labsystems, Helsinki, Finland) with excitation at 480 nm and emission at 538 nm. Synaptic

activity in dissociated hippocampal neurons was analyzed as described [17].

***Analysis of synaptic vesicle cycling and immunocytochemistry.*** Polyclonal sera used in these studies were obtained from rabbits and goats immunized with a synthetic peptide ($NH_2$-MVSASHPEALAAPVTTVATLVPHC-COOH) corresponding to the intravesicular N-terminal portion of synaptotagmin-I [17,32,33]. These antibodies were affinity-purified as earlier described [17]. For the present experiments, anti-synaptotagmin antibodies were added to the culture medium (supplemented with 25 μM APV (2-amino-5-phosphonovalerate) to block NMDA receptor-mediated currents) at a final dilution of 1:100-1:200, and the hippocampal cell cultures were field-stimulated with platinum wires at 0.5 Hz for 10 minutes (at 37 °C and 95% air, 5% $CO_2$). At the end of this 10-minute stimulation, cultures were washed extensively with oxygenated Tyrode solution (37 °C) containing 119 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 4 mM $MgCl_2$, 30 mM glucose, 25 μM APV, and 25 mM HEPES, pH 7.4, and then fixed with 4% paraformaldehyde. Fixed cells were permeabilized in blocking solution (0.4% (wt/vol) BSA, and 0.4% (wt/vol) saponin in 120 mM phosphate buffer), incubated with primary antibodies (monoclonal antibodies against the cytosolic domain of synaptotagmin-I) dissolved in blocking solution (2 hours, room temperature), and, after an extensive wash with blocking solution, incubated at room temperature with species-specific fluorochrome-conjugated secondary antibodies (1 hour, room temperature). Fluorescence images were captured with a one-photon confocal microscope (Zeiss). Quantification of the number of synapses and of the vesicular uptake of anti-synaptotagmin-I antibodies was done using a computer program written in house [32,33]. Synapses were first identified based on intensity and shape parameters, and then fluorescence intensities from internalized antibodies were measured on a different excitation/emission channel from the same areas. Intensity values were determined near the center of individual boutons and used for analysis if they exceeded the SD of the background fluorescence by > 3 times.

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# ISOLATION AND BASIC CHARACTERIZATION OF TEMPERATURE-SENSITIVE MUTANTS FROM SEMLIKI FOREST VIRUS

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Sixteen temperature-sensitive mutants were isolated from N-methyl-N'-nitro-N-nitrosoguanidine treated Semliki Forest virus. Seven of the mutants were unable to synthesize virus specific RNA at the nonpermissive temperature (39° C) and were classified as RNA⁻ mutants; as evidenced by temperature shift-up experiments they had defects only in early functions. Two mutants, designated RNA±, made less virus specific RNA than the wild type virus. Six RNA⁺ mutants synthesized 42 S and 26 S RNA at 39° C in almost the same amounts and ratio as the wild type. Only one of them, ts-3, was unable to form the viral 140 S nucleocapsid at the nonpermissive temperature. One of the RNA⁺ mutants, ts-1, made a higher proportion of 42 S RNA, presumably at the expense of 26 S RNA, both at 27° C and at 39° C. The synthesis of 42 S RNA and 26 S RNA in the Semliki Forest and Sindbis wild type virus infected cells was temperature dependent. There was an increased synthesis of 42 S RNA compared to 26 S RNA at 39° C, the situation being reversed at 27° C.

The main project of our group is to study the structure and replication of Semliki Forest virus, SFV (2, 11, 22, 23, 24, 26, 27 and 28), an arbo A virus, which belongs to the recently established togavirus group (1, 31). Because of the great value of the conditional lethal mutants in these studies, the isolation of temperature-sensitive (ts) mutants from SFV was begun in a manner similar to that reported earlier for the closely-related Sindbis virus (3).

The Sindbis virus mutants were first classified into five non-overlapping complementation groups (4) two of which were composed of mutants unable to produce viral RNA at the restrictive temperature. The other three complementation groups were linked to defects either in envelope, nucleocapsid, or a hypothetical maturation protein (4, 5, 6, 32). Later the RNA negative mutants were reclassified into three complementation groups (14).

The biochemical properties of the Sindbis virus mutants have been studied intensively (5, 6, 17, 18, 19, 20, 32). Temperature-sensitive mutants have also been isolated from Semliki Forest virus but their biochemical properties have been investigated less intensively (15, 29).

In this paper we report the isolation and basic characterization of 16 ts mutants obtained from nitrosoguanidine treated SFV.

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The characterization of the mutants is biochemical rather than genetic because of the inherent difficulties in the recombination and complementation of these RNA viruses since the proteins are made by proteolytic cleavage of large precursor proteins (18, 19, 20). In an earlier report we showed that the cleavage of the precursor (NVP 68) of the envelope protein $E_2$ is inhibited in cells infected with ts-1 mutant (23). A more detailed report on the proteins synthesized by this and our other mutants at the nonpermissive temperature is in preparation.

## MATERIALS AND METHODS

### *Virus and Cell Cultures*

SFV, prototype strain (10), was cloned three times before use both as the wild type virus and the source of the mutants. Other viruses used were a prototype strain of SFV received from the American Type Culture Collection and Sindbis virus, prototype EgAr 339, received from Dr. *J. Casals* (Yale Arbovirus Research Unit). Leukosis free secondary chick embryo fibroblasts (CE) received from this laboratory's leukosis free flock were grown on 80 or 50 mm petri dishes, 250 ml bottles (Falcon Plastics) or on plastic well plates (Lindbro). Growth medium consisted of Eagles minimal essential medium (MEM) supplemented with 5 per cent inactivated calf serum and 10 per cent tryptose phosphate broth (Difco). 39° C was selected as the nonpermissive temperature and the incubator was kept in a 37° C hot room. The permissive temperature was 27° C. Both incubators were flushed constantly with 5 per cent $CO_2$.

### *Plaque Assay*

For virus cloning and mutant isolation the plaque assay was performed using an agar overlay containing 0.9 per cent Bacto agar (Difco) in medium 199 supplemented with 5 per cent inactivated calf serum. When the plaque forming units (PFU) were determined 0.5 per cent carboxy methyl cellulose was used to solidify the overlay medium (30). The plaque counts were obtained after 2 days at 39° C or after 4 days at 27° C.

### *One Cycle Growth Experiments*

Cells were infected with 10 or 50 PFU/cell at the desired temperature. After 1 h adsorption the inoculum was removed and the cells were washed three times with prewarmed Hanks' balanced salt solution. In some experiments the cells were rewashed 4 h post infection. The medium used was MEM containing 0.2 per cent bovine serum albumin (BSA, Armour) and actinomycin D, 2 μg/ml as indicated. The released virus was harvested 8 (39° C) or 16 (27° C) h post infection. The cell debris was removed from the culture medium by low speed centrifugation and the supernatant was used for hemagglutination and infectivity assays.

### *Mutagen Treatment*

N-methyl-N'-nitro-N-nitrosoguanidine treatment (100 μg/ml in 0.01 M tris buffer, pH 7.6) of the wild type virus was performed as described by *Burge & Pfefferkorn* (3), except that the treatment period was 30 min at 20° C and that part of the sample was diluted immediately to $10^{-7}$ for plaque assay at 27° C and part was dialyzed over night against phosphate buffered saline (PBS) containing 0.5 per cent BSA, before the plaque assay. The infectivity recovered in the undialzed and in the dialyzed sample was 6.7 per cent and 2.8 per cent respectively.

### *Isolation of Mutants*

Well isolated plaques formed at 27° C under agar were collected and virus was eluted in 1 ml of PBS containing 0.5 per cent BSA. The ability of the eluted virus to grow at 39° C was tested using less than 1 PFU/cell in monolayers in plastic well plates. The cytopathic effect (CPE) was recorded at 24 h. The growth of those eluates with no CPE at 39° C was similarily tested at 27° C and the CPE was recorded at 48 h. Those eluates which showed CPE at 27° C only were assayed for infectivity at both temperatures. When the PFU titre at 27° C was 100 fold or more above that at 39° C, three well isolated plaques were collected from the 27° C assay plate. This was the second cloning of the mutants. To obtain the primary stock one plaque was grown in a cell culture for 48 h at 27° C at an input multiplicity of infection of less than 1 PFU/cell. From these stocks the working stocks were grown for one further passage only at a multiplicity of infection 1 to 100 at 27° C.

### *Selection of Mutants*

The primary stock was assayed at 39° C and at 27° C. When the PFU ratio at 39° C/27° C (plating efficiency) was $10^{-4}$ or lower the mutant was accepted and the leak yield determined. The cultures were infected with 10 PFU/cell at both temperatures. Culture fluids were collected at 8 h (39° C) and 16 h (27° C) and the infectivity of both harvests was determined by plaque assay at 27° C. Those mutants with a leak yield (virus yield at 39° C/virus yield at 27° C) of $10^{-3}$ or below were finally accepted for further studies.

*Temperature Shift-up Experiments*

Duplicate plates were infected at 27° C using 10 PFU/cell as described before. At 5 h post infection the culture medium was removed and replaced with fresh medium at 39° C and the incubation was continued at 39° C for another 5 h after which the culture fluid was harvested and the infectious virus formed was measured by plaque assay at 27° C. Another set of cultures used as controls was infected and maintained at 39° C for 8 h.

*Hemagglutination Titration*

The titration was performed at room temperature or at 39° C as indicated using 0.2 per cent goose erythrocytes at pH 5.8 (7).

*RNA Synthesis*

Confluent monolayers were infected at 50 PFU/cell in the presence of 2 μg/ml of actinomycin D as described above. At the times indicated $^3$H-uridine was added in 2 ml of fresh medium containing 20 μCi of $^3$H-uridine (24–31.4 Ci/mmol, Amersham) and the incubation was continued for two more hours. After the pulse the cells were washed once with PBS and once with RSB-Na (0.01 M Tris pH 7.4, 0.01 M NaCl, 0.0015 M $MgCl_2$) or with TSE (0.01 M Tris pH 7.4, 0.15 M NaCl, 0.001 M EDTA-Na). The monolayers were then disrupted in 2 per cent sodium dodecyl sulphate (SDS) in the final wash buffer, and the cell lysate homogenized by passing through a small syringe needle. The acid insoluble radioactivity was measured from aliquots after precipitation with 5 per cent TCA (9). Sedimentation analysis was carried out on 15–30 per cent (w/w) sucrose gradients made in RSB-Na or TSE containing 0.1 per cent SDS in 18 ml capacity tubes (Spinco SW27 rotor). Centrifugation was for 10 or 12 h at 24,000 rev/min at 22° C. After centrifugation 0.5 ml fractions were collected from below and acid insoluble radioactivity was measured as described above.

*Nucleocapsid Analysis*

Cells infected with the mutants and kept at 39° C were exposed, from 5 to 5½ h post infection to $^{14}$C-amino acids, 20 μCi/dish (57 mCi/mAtom carbon) in MEM containing 1/10 of the normal amount of amino acids or 100 μCi/dish of $^3$H-lysine (250 μCi/mmol) in MEM from which lysine was omitted. After the pulse the medium was removed and the cells were washed twice with MEM containing ten times the normal amino acids or lysine concentration. The cells were maintained in the chase medium for a further 30 min at 39° C. At the end of the chase the cells were washed with cold PBS, swollen in RSB-Na and disrupted in a Dounce homogenizer. To the homogenate Triton X-100 was added to a final concentration of 1 per cent and the nuclei were pelleted at 250 *g* for 5 min. The cytoplasm was layered over 34 ml 15–30 per cent (w/w) sucrose gradients made in 0.05 M Tris pH 7.4, 0.1 M NaCl 0.0015 M

TABLE 1. *Basic Characteristics of the 16 Temperature-Sensitive Mutants of SFV*

| Virus | RNA phenotype | Reversion frequency | Leak yield | RNA synthesis at 39° C per cent of wild type | Nucleocapsid formation |
|---|---|---|---|---|---|
| ts-1 | RNA$^+$ | $<3.6 \times 10^{-6}$ | $1.7 \times 10^{-6}$ | 102 | + |
| ts-2 | RNA$^+$ | $<3.5 \times 10^{-5}$ | $9.1 \times 10^{-6}$ | 66 | + |
| ts-3 | RNA$^+$ | $<1.1 \times 10^{-4}$ | $<5.9 \times 10^{-6}$ | 82 | — |
| ts-4 | RNA$^-$ | $<1.4 \times 10^{-6}$ | $2.1 \times 10^{-6}$ | 0.9 | ND |
| ts-5 | RNA$^+$ | $<4.0 \times 10^{-6}$ | $6.5 \times 10^{-4}$ | 72 | + |
| ts-6 | RNA$^-$ | $<2.8 \times 10^{-6}$ | $1.5 \times 10^{-5}$ | 1.1 | ND |
| ts-7 | RNA$^+$ | $<5.5 \times 10^{-5}$ | $1.5 \times 10^{-4}$ | 57 | + |
| ts-8 | RNA$^-$ | $<6.1 \times 10^{-7}$ | $1.5 \times 10^{-6}$ | 1.2 | ND |
| ts-9 | RNA$^-$ | $<2.4 \times 10^{-6}$ | $<3.7 \times 10^{-6}$ | 0.9 | ND |
| ts-10 | RNA± | $<2.2 \times 10^{-5}$ | $<2.0 \times 10^{-5}$ | 7.2 | ND |
| ts-11 | RNA$^-$ | $<6.7 \times 10^{-7}$ | $8.0 \times 10^{-5}$ | 4.4 | ND |
| ts-12 | RNA$^-$ | $<5.9 \times 10^{-7}$ | $1.0 \times 10^{-4}$ | 1.3 | ND |
| ts-13 | RNA± | $<9.5 \times 10^{-7}$ | $2.6 \times 10^{-6}$ | 19 | — |
| ts-14 | RNA$^-$ | $2.4 \times 10^{-5}$ | $1.4 \times 10^{-4}$ | 3.3 | ND |
| ts-15 | RNA$^+$ | $<1.0 \times 10^{-7}$ | $<6.2 \times 10^{-7}$ | 44 | + |
| ts-16 | RNA$^+$ | $<6.9 \times 10^{-6}$ | $5.9 \times 10^{-6}$ | 78 | + |

ND = not done.

$MgCl_2$ and centrifuged for 3 h at 4° C in a Spinco SW27 rotor at 25,000 rev/min. One ml fractions were collected from below and the acid insoluble radioactivity determined. In some cases double labelling was carried out using in addition to $^{14}C$-amino acids also $^{3}H$-uridine (100 μCi/dish). Except for washing, no attempt was made to stop the incorporation of uridine after the pulse.

## RESULTS

### *Provisional Characterization of the Mutants*

Altogether 340 plaques were collected from cloned, NTG treated Semliki Forest virus. A total of 16 ts-mutants with a reversion frequency lower than $10^{-4}$ and leak yield below $10^{-3}$ were obtained and coded ts-1 to ts-16 (Table 1). The mutants gave essentially the same yield as the wild type virus at 27° C which reached its maximum at about 16 h p.i. (Fig. 1). The working stocks showed the same reversion frequencies and leakiness as the primary stocks.

Hemagglutination titration of the wild type and all mutants after growth at 27° C were carried out both at room temperature and at 39° C. In every case including the wild type, the titer obtained at room temperature was 8 fold higher than that at 39° C. This indicates that none of the mutants have temperature-sensitive defects which affect the HA reaction. Nor did any of the mutants release hemagglutinins into the medium at 39° C, indicating that noninfectious, hemagglutinating particles were not produced.

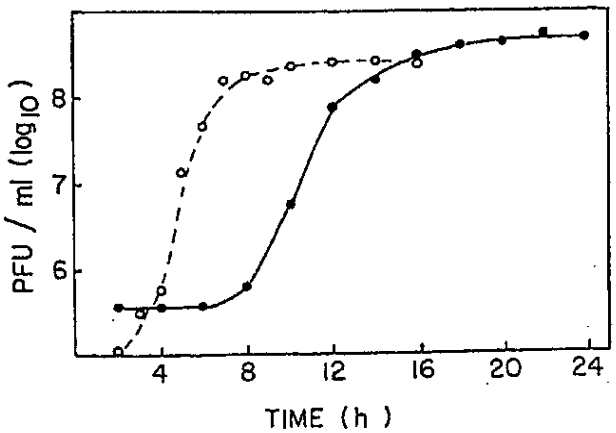


*Fig. 1.* One step growth curves of wild type SFV in secondary chick embryo fibroblast monolayers. At each point the culture fluid from duplicate cultures on 50 mm petri dishes (infected with 50 PFU/cell at zero time) was pooled and PFU titer was determined at 27° C. O——O growth at 39° C, —— growth at 27° C.

The initial determination of the RNA phenotype was done at 39° C by giving a two hour pulse of $^{3}H$-uridine to the infected cells in the presence of actinomycin D. Under these conditions only the viral RNA is labelled (9). In the wild type infected cells the acid insoluble radioactivity was 50 to 100 times that of the uninfected control cells. Seven of the mutants (ts-1, ts-2, ts-3, ts-5, ts-7, ts-15, ts-16) incorporated $^{3}H$-uridine very efficiently, 40 to 100 per cent of the wild type value and were therefore classified RNA positive ($RNA^+$) mutants. The RNA negative ($RNA^-$) phenotype was assigned to those mutants (ts-4, ts-6, ts-8, ts-9, ts-12) giving only 1 to 2 per cent of the wild type incorporated radioactivity (Table 1). Four mutants (ts-10, ts-11, ts-13, ts-14) incorporated $^{3}H$-uridine clearly less than wild type virus but differed also from the background.

Temperature shift-up experiments were carried out with all the clearly RNA negative mutants and with those showing some $^{3}H$-uridine incorporation. The infected cells were first kept at 27° C for 5 h and thereafter shifted to 39° C for a further 5 h. Under these conditions those mutants which have defects only in functions needed early in the replication should grow normally. All the $RNA^-$ mutants replicated in the shifted cultures (Table 2). The ts-2 a $RNA^+$ mutant did not replicate after the shift up to 39° C. Two mutants ts-11 and ts-14 showing little RNA synthesis (Table 1), behaved exactly like the $RNA^-$ mutants and were therefore included in the $RNA^-$ group. Two mutants ts-10 and ts-13, capable of significantly higher RNA synthesis (7 and 19 per cent respectively) did not yield infectious virus in the shifted cultures. These mutants were classified into an intermediate group designated as $RNA^{\pm}$ (Table 1).

The temperature shift-down experiment in which the infected cultures were incubated for 4 h at 39° C and thereafter shifted to

TABLE 2. *Temperature Shift-up Experiment*

| Virus | RNA phenotype | Plaque forming unit per cell | |
|---|---|---|---|
| | | 8 h at 39° C | 5 h at 27° C + 5 h at 39° C |
| ts-4 | RNA⁻ | 0.005 | 16 |
| ts-6 | RNA⁻ | 0.005 | 83 |
| ts-8 | RNA⁻ | 0.07 | 110 |
| ts-9 | RNA⁻ | 0.003 | 3.8 |
| ts-11 | RNA⁻ | 0.04 | 190 |
| ts-12 | RNA⁻ | 0.4 | 130 |
| ts-14 | RNA⁻ | 0.7 | 240 |
| ts-10 | RNA± | 0.001 | 0.003 |
| ts-13 | RNA± | 0.001 | 0.002 |
| ts-2 | RNA⁺ | 0.008 | 0.004 |
| wild type | RNA⁺ | 180 | 140 |

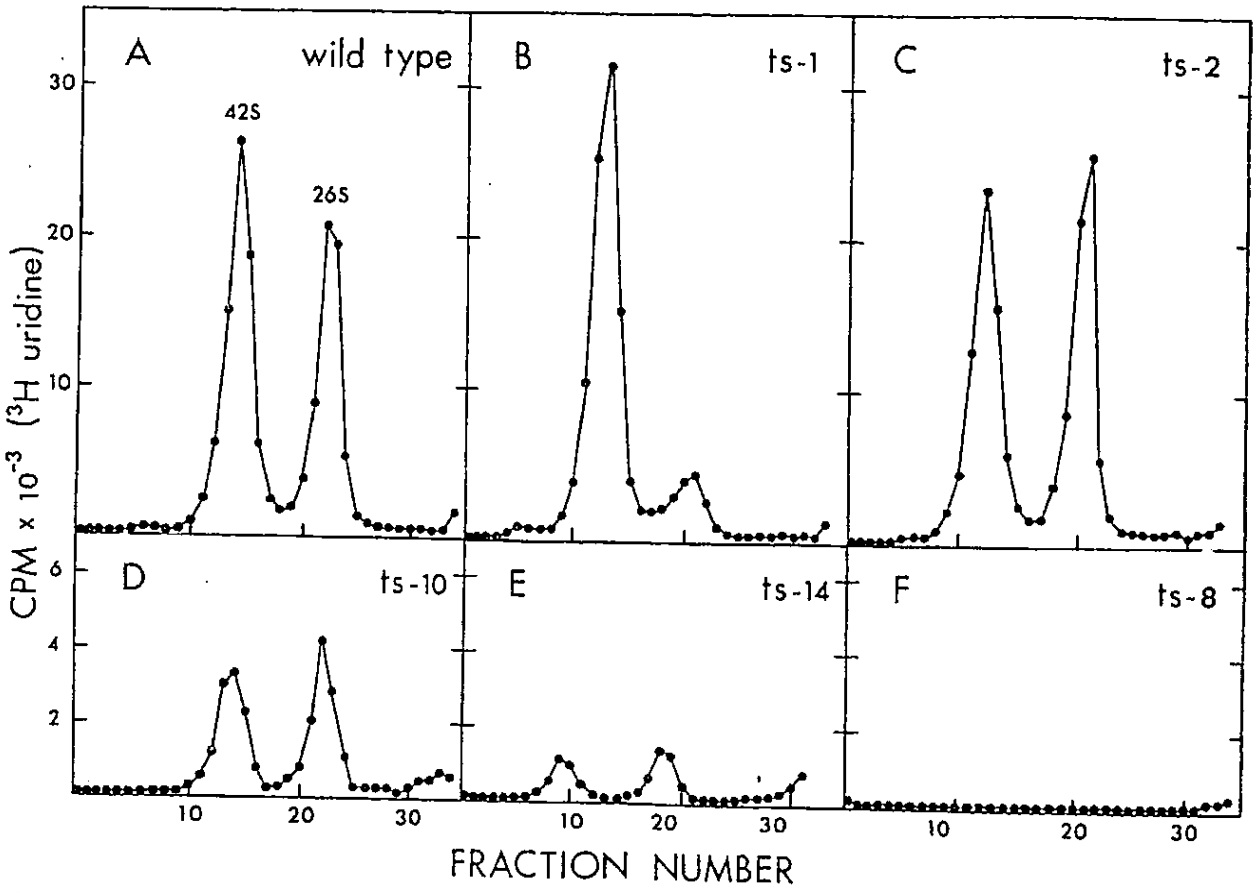


*Fig. 2.* Sucrose gradient analysis of wild type (A), ts-1 (B), ts-2 (C), ts-10 (D), ts-14 (E) and ts-8 (F) mutants of Semliki Forest virus grown at 39° C. Two petri dishes of secondary CE cells infected with 50 PFU/cell and maintained in MEM + 0.2 per cent BSA + 2 μg/ml of actinomycin D were pulsed from 3 to 5 h p.i. with 10 μCi/ml of ³H-uridine. At the end of the pulse cells were washed, taken into RSB-Na containing 2 per cent SDS, disrupted and analyzed in 15–30 per cent sucrose made in RSB-Na containing 0.1 per cent SDS as described in Materials and Methods. Centrifugation was for 10 h at 24,000 rev/min in SW27 rotor (18 ml tubes) at 22° C. S-values were determined using ribosomal 28 S and 18 S RNA absorbancy markers: Bottom at left.

TABLE 3. *Synthesis of 42 S and 26 S RNA in Semliki Forest Virus ts-mutant Infected Cells at 39° C*

| Virus | RNA phenotype | $^3$H-uridine incorporated into $10^6$ cells | | | 42S/26S RNA ratio |
|---|---|---|---|---|---|
| | | Total CPM × $10^{-3}$ | Per cent in 42 S RNA | Per cent in 26 S RNA | |
| ts-1 | RNA$^+$ | 103 | 69.7 | 9.0 | 7.7 |
| ts-2 | RNA$^+$ | 77.5 | 41.0 | 35.6 | 1.2 |
| ts-3 | RNA$^+$ | 85.6 | 41.1 | 34.9 | 1.2 |
| ts-5 | RNA$^+$ | 64.4 | 51.3 | 25.6 | 2.0 |
| ts-7 | RNA$^+$ | 65.6 | 46.2 | 28.8 | 1.6 |
| ts-10 | RNA± | 7.3 | 36.3 | 32.7 | 1.1 |
| ts-11 | RNA$^-$ | 4.5 | 40.0 | 22.3 | 1.8 |
| ts-13 | RNA± | 17.8 | 49.8 | 24.9 | 2.0 |
| ts-14 | RNA$^-$ | 3.0 | 31.6 | 28.9 | 1.1 |
| ts-15 | RNA$^+$ | 42.2 | 42.6 | 33.8 | 1.3 |
| ts-16 | RNA$^+$ | 71.8 | 48.3 | 27.8 | 1.7 |
| wild type | RNA$^+$ | 90.0 | 47.0 | 31.8 | 1.5 |

27° C for a further 4 h was carried out with ts-10 and ts-13. The yields of the shifted cultures were 1.4 and 0.002 PFU/cell for ts-10 and ts-13 respectively. According to these results both these mutants seem to have defects in both the early and late functions of replication.

### *Synthesis of the Virus Specific RNAs at 39° C*

The RNA species synthesized by the mutants in actinomycin D treated cells at the nonpermissive temperature were determined using a two hour pulse of $^3$H-uridine beginning 3 h post infection. The sucrose gradient profiles of the RNAs of wild type virus and some of the mutants are shown in Fig. 2. Under these conditions, in arbo A virus infected cells, there are two major virus specific single-stranded RNAs formed, which sediment at 42 S and 26 S (8). The former is the viral genome and the latter represents gene duplication of 1/3 of the 42 S RNA (21). The replicative intermediate sedimenting between 20 and 29 S (9) and the single stranded 20 S, 33 S and 38 S RNAs also found in SFV infected cells account for only a small amount of the total RNA (13, 16). Repeated experiments with the wild type virus have shown that at most 10 per cent of the RNA sedimenting at 26 S is resistant to pancreatic RNase (5 μg/ml 30 min at 37° C) due to a contamination by replicative intermediates. Most of this RNA represents, however, genuine single stranded 26 S RNA and is referred to as such.

The acid insoluble radioactivity sedimenting at 42 S and 26 S was calculated from the sucrose gradient fractions and compared to the total acid insoluble radioactivity recovered from the gradient. The results for all RNA$^+$, RNA±, and also for two RNA$^-$ mutants showing slight RNA synthesis (Table 1) are expressed per $10^6$ cells and given in Table 3.

For the wild type a value of 1.5 for the 42 S/26 S RNA ratio was obtained. In similar two hour pulses with $^3$H-uridine the value varied from 1.5 to 2.5 (4 experiments). All but one of the mutants tested had 42 S/26 S RNA ratios between 1 and 2. The only exception was ts-1 which synthesized considerably less RNA sedimenting at 26 S, although the total amount of RNA synthesized was not reduced. About 1½ times more 42 S RNA was made than in wild type infected cells, suggesting that the reduced synthesis of 26 S RNA was compensated for by an increased synthesis of 42 S RNA and resulting in a 42 S/26 S RNA ratio of 7.7 in this particular experiment. The overall variations in the

RNA ratios was 6.1 to 10.7 (4 experiments). About 20 per cent of the radioactivity at 26 S was resistant to pancreatic RNase (5 μg/ml 30 min at 37° C) suggesting that the actual amount of 26 S RNA was less than that indicated in Table 3.

### *Temperature Dependence of the 42 S/26 S RNA Ratio*

Because the ts-1 mutant was clearly deficient in the synthesis of the 26 S RNA at the nonpermissive temperature, RNA synthesis at both 27° C and 39° C was studied more closely. In these experiments two other mutants, ts-2 and ts-3, as well as two different strains of SFV and one strain of Sindbis were included. A two hour pulse of $^3$H-uridine was given at 3 h and 6 h post infection at 39° C and 27° C respectively and the total virus specific cellular RNAs were analyzed by sucrose gradient centrifugation in TSE (Table 4). 42 S and 26 S RNA accounted for 70–90 per cent of the total acid insoluble radioactivity at both temperatures. A striking difference in the 42 S/26 S RNA ratio at 27° C and 39° C was observed with all the viruses tested. At the lower temperature more 26 S RNA and less 42 S RNA was synthesized than at the higher temperature. The increased amount in 26 S RNA at 27° C could not be accounted for by contamination with the replicative intermediate since the level of RNase resistant material remained at 10 per cent of the total again.

The 42 S/26 S RNA ratio for the same stock virus varied to some extent, but the difference obtained between the two temperatures was always 3 fold or greater. The ts-2 and ts-3 mutants showed wild type RNA synthesis patterns at 39° C and 27° C. The ts-1 mutant produced more 42 S RNA at both temperatures than any of the other viruses studied. It still exhibited the same difference in ratios between the two temperatures as the other mutants and the wild type virus. Several temperature shift-up and -down experiments were carried out with ts-1 and the wild type: All of them confirmed the temperature dependence of the 42 S/26 S RNA ratio for both mutant and wild type. Inhibition of protein synthesis by cycloheximide (100 μg/ml) before the cultures were shifted down gave the same 42 S/26 S RNA ratio as in the absence of cycloheximide. This shows that the alteration in RNA synthesis does not require protein synthesis.

### *Nucleocapsid Formation*

In SFV infected cells the viral nucleocapsid which consists of the 42 S RNA genome and about 240 identical capsid proteins, MW 33,000 (10, 12), is formed from a polysomal precursor (27) within 15 min (25). The ability of the mutants to form viral nu-

TABLE 4. *Effect of the Temperature on the Synthesis of Semliki Forest Virus 42 S and 26 S RNAs in CE Cells*

| Virus strain | Temperature during virus growth and period of labelling with $^3$H-uridine | | | | | |
|---|---|---|---|---|---|---|
| | 39° C pulse 3 – 5 hours p.i. | | | 27° C pulse 6 – 8 hours p.i. | | |
| | Per cent in 42 S RNA | Per cent in 26 S RNA | 42 S/26 S | Per cent in 42 S RNA | Per cent in 26 S RNA | 42 S/26 S |
| Wild type (cloned) | 57.0 | 24.3 | 2.3 | 31.9 | 42.5 | 0.75 |
| ts-1 | 73.8 | 6.9 | 10.7 | 57.5 | 21.9 | 2.62 |
| ts-2 | 55.2 | 24.6 | 2.2 | 27.7 | 41.2 | 0.67 |
| ts-3 | 55.2 | 25.5 | 2.2 | 26.9 | 49.3 | 0.55 |
| SFV prototype strain 1 | 65.9 | 18.9 | 3.5 | 22.3 | 59.8 | 0.37 |
| SFV prototype strain 2 | 54.1 | 23.9 | 2.3 | 24.1 | 55.4 | 0.44 |
| Sindbis | 38.9 | 37.3 | 1.0 | 18.6 | 55.0 | 0.34 |

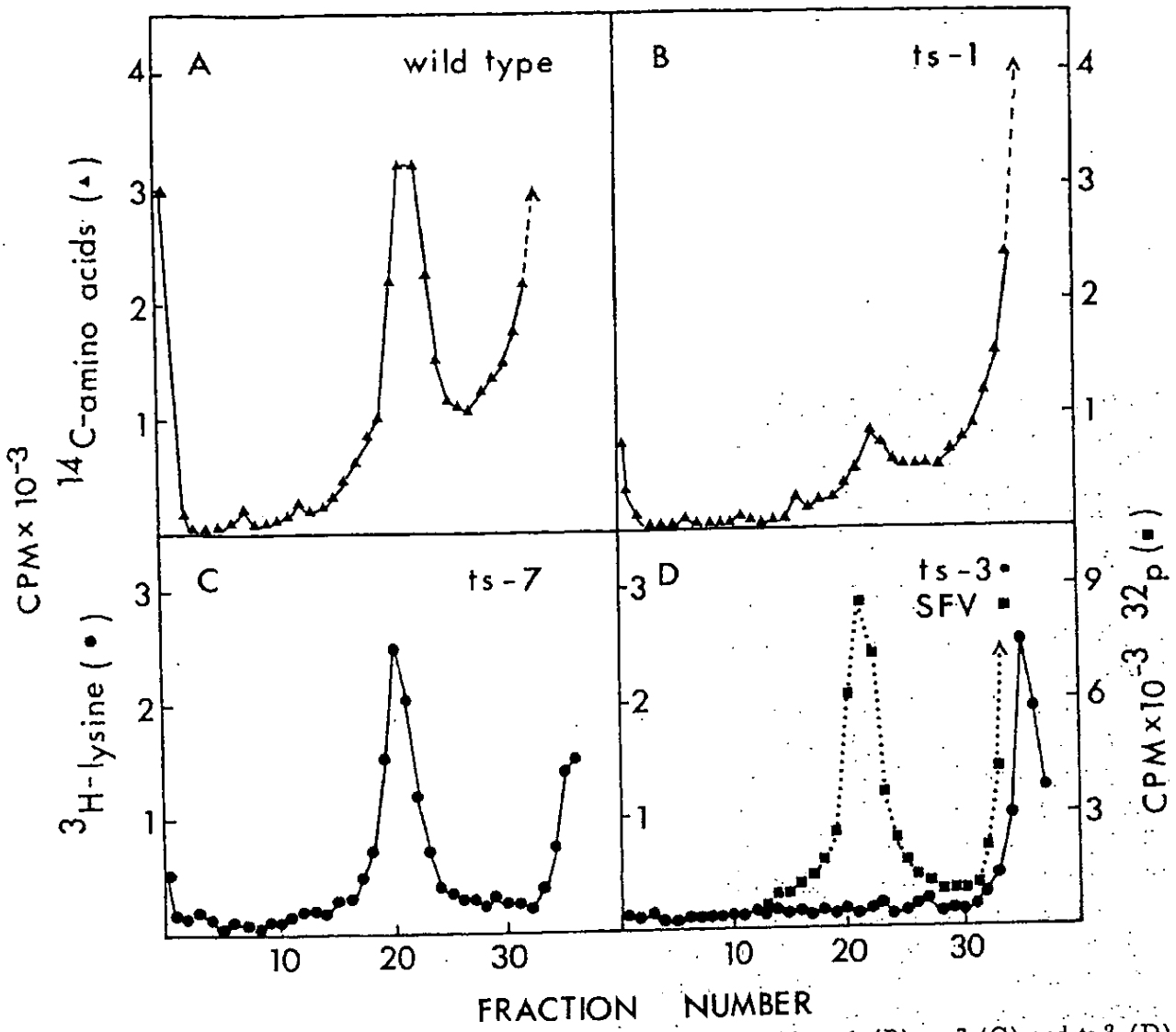


*Fig. 3.* Nucleocapsid synthesis in CE cells infected with wild type (A), ts-1 (B), ts-7 (C) and ts-3 (D) mutants of Semliki Forest virus. Infected cells were pulsed with $^{14}$C-amino acids (10 μCi/ml) or with $^{3}$H-lysine (50 μCi/ml) for 30 min at 5 h p.i. followed by a 30 min chase. The preparation of the cytoplasmic extract in 1 per cent Triton X-100 was as described in Materials and Methods. Centrifugation was for 3 h at 25,000 rev/min in SW27 rotor (38 ml tubes) at 4° C. SFV labelled with $^{32}$P was treated with 1 per cent Triton X-100 to release the nucleocapsid and centrifuged in a separate tube. $^{14}$C: ▲, $^{3}$H: ●, $^{32}$P: ■. Sedimentation was from right to left.

cleocapsid at the nonpermissive temperature was tested by giving a 30 min pulse of $^{14}$C-amino acids of $^{3}$H-lysine to the infected cells at 5 h post infection in the presence of actinomycin D. After the pulse, nonradioactive amino acids were added and the incubation was continued for 30 min at 39° C. The cytoplasm was collected and analyzed in 15 to 30 per cent sucrose gradient after solubilization of the membranes by 1 per cent Triton X-100. Under these conditions radioactive material sedimenting with a peak at about 140 S consists almost exclusively of nucleocapsids (27). In Fig. 3 sucrose gradient profiles of the cytoplasmic nucleocapsid of wild type virus, ts-1, ts-3, and ts-7 are presented. In the wild type infected cells 54 per cent of the sedimenting (>20 S) $^{14}$C-amino acid label was found between 120 S and 160 S. The corresponding figure for ts-7, one of the nucleocapsid positive mutants, was 54 per cent with $^{14}$C-amino acids and 63 per cent with $^{3}$H-lysine label. Ts-5 was shown to be almost as efficient a producer of nucleo-

capsid (54 per cent $^{3}$H-lysine label sedimented between 120–160 S). Mutants ts-1 ($^{14}$C:43 per cent) ts-2 ($^{3}$H:36 per cent) ts-15 ($^{14}$C:31 per cent) and ts-16 ($^{14}$C:45 per cent) were somewhat less efficient in the capacity to form nucleocapsid. These were, however, classified as nucleocapsid positive mutants. Ts-3, clearly a RNA positive mutant, did not show any nucleocapsid peak (Fig. 3) nor did ts-13 which is a RNA± mutant. Double labelling with $^{3}$H-uridine and $^{14}$C-amino acids was performed with mutants ts-1, ts-2, ts-13, ts-15, and ts-16. The RNA from the 140 S peak was analyzed. In all cases it was largely (>70 per cent) 42 S RNA.

## DISCUSSION

In the isolation of temperature-sensitive mutants from Semliki Forest virus reported in this paper, fairly stringent criteria were used for the back mutation frequency ($10^{-4}$) and for the leakiness ($10^{-3}$), in order to select stable mutants suitable for biochemical studies. This may have led us to select some double mutants. Their proportion should, however, be low since the same conditions and criteria were used as for the Sindbis virus mutants of Burge and Pfefferkorn most of which were single step mutants as shown by their ability to form complementation groups. Two of our mutants, ts-10 and ts-13, showed defects both in early and late functions in replication (Table 2) and are probably double mutants.

The main characteristics of the mutants are given in Table 1. Five of them are unable to synthesize detectable amounts of viral RNA at the nonpermissive temperature and showed only early function defects in temperature shift-up experiment. Two mutants, ts-11 and ts-14, which showed some RNA synthesis at the nonpermissive temperature, were included in the RNA$^{-}$ group since they behaved in the shift-up experiment like RNA negative mutants. Two other mutants, ts-10 and ts-13, synthesized somewhat more RNA. Neither of them replicated after a shift to the nonpermissive temperature thus showing a late function defect in addition to the obvious impairement of RNA synthesis. Since a considerable amount of RNA (7 and 19 per cent for ts-10 and ts-13 respectively) is formed at 39° C one would except at least some infectious virus to be made in cultures shifted down to the permissive temperature. This turned out to be so for ts-10. Interestingly enough, ts-13 did not show increased virus production in the shifted cultures despite its higher RNA synthesis. It is possible that the RNA formed at high temperature is either unable to direct viral protein synthesis or cannot form virus particles even at the lower temperature.

All the RNA$^{+}$ and RNA± mutants except ts-1 synthesized both 42 S and 26 S RNA at the nonpermissive temperature in the same ratio as the wild type virus. Ts-1 showed a clear increase in 42 S and decrease in 26 S RNA synthesis. When the temperature dependence of this mutation was studied in more detail an interesting property of the wild type was noticed. The 42 S/26 S RNA ratio was found to be temperature dependent; 2–3 times more 42 S RNA was produced at 39° C than at 27° C. This increased 42 S RNA synthesis was seemingly compensated by reduced synthesis of 26 S RNA. This observation was confirmed with two different strains of SFV and also with Sindbis virus. The temperature dependent change of the ts-1 42 S/26 S RNA ratio was of the same order of magnitude as that of the wild type. Anyhow, it is obvious that the increased capacity of ts-1 to produce 42 S RNA at both temperatures is due to a mutation. The mutation causing more 42 S to be synthesized by ts-1 appears to be temperature independent and was detected only because ts-1 has another temperature sensitive mutation, namely the inability to cleave the envelope protein precursor NVP 68 into $E_2$ (23). In Sindbis virus infected cells the 42 S/26 S RNA ratio can be altered by inhibiting the protein synthesis 1½ h post infection as shown by *Scheele & Pfefferkorn* (17). As a result much less 26 S RNA is produced. The authors suggest that a specific protein is

needed for the synthesis of 26 S RNA. We think that this "26 S protein" is altered in our ts-1 due to a mutation. In the Sindbis ts-24 a similar mutation has been suggested (17).

The temperature dependence of the 42 S/26 S RNA ratio and the properties of ts-1 could be explained by assuming that the normal "26 S protein" binds reversibly to the replicase or possibly to the template. This would allow transcription of either 26 S RNA or the whole 42 S RNA. The binding is more stable at the lower temperature resulting in a pronounced 26 S RNA synthesis while the reverse is true at the higher temperature. A mutation affecting the "26 S protein" could result in reduced affinity for the acceptor as would be the case with our mutant ts-1 and Sindbis mutant ts-24.

The temperature dependence of the association constant of this protein may well be connected with the ability of arbo A viruses to grow at low (arthropods) as well as at high (mammalians and birds) temperatures.

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Key words: *SFV/mouse embryos/temperature-sensitive mutants*

# Isolation and Preliminary Characterization of Semliki Forest Virus Mutants With Altered Pathogenicity for Mouse Embryos

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SUMMARY

Four temperature-sensitive (*ts*) mutants of the A7 strain of Semliki Forest virus (SFV) have been isolated. All mutants were defective in RNA synthesis at the restrictive temperature (39 °C) compared to the permissive temperature (30 °C). Since the body temperature of mice fluctuates between 37 °C and 39 °C, multiplication was also examined at 37 °C; only the multiplication of *ts*4 was restricted. After intraperitoneal infection of 8-day pregnant mice, the wild-type induced rapid abortion. *Ts*4 and *ts*26 had no effect on embryonic development. Litters born to *ts*4-infected mothers developed no postnatal immunity whereas 50% of litters from *ts*26-infected mothers were immune. Unlike the wild-type, *ts*14 induced the same or higher virus titres in placental tissue in most mice than in foetal tissue. *Ts*22 and *ts*14 induced a range of development defects, including developmental arrest, mummification, abortion and postnatal death. Most surviving offspring were immune. Although *ts*4 induced no viraemia, *ts*14, *ts*22 and *ts*26 induced a lower titre but longer lasting viraemia than the wild-type. It is concluded that infections of pregnant mice with *ts*14 and *ts*22 in particular are good models for analysis of the mechanism of virus-induced developmental defects.

## INTRODUCTION

Embryonic and foetal infections due to togaviruses have been reported both in humans for viruses such as rubella (Gregg, 1941; Horstmann, 1976) and in domestic animals for viruses such as bovine viral diarrhoea (Done *et al.*, 1980) and border disease (Barlow *et al.*, 1980). In general, the outcome of infection depends on the stage of development at the time of infection, with the most serious consequences following exposure during early gestation (Dudgeon, 1969; Johnson, 1982). Foetuses may be aborted, stillborn, mummified or survive with congenital abnormalities. Some clinically normal offspring develop signs of disease later in life (Barlow *et al.*, 1980; Dudgeon, 1969; Van Oirschot, 1983).

Models of embryonic infection based on infection of pregnant mice have been developed for the flaviviruses St Louis encephalitis (Anderson & Hanson, 1975) and Japanese encephalitis (Mathur *et al.*, 1981). The A7 strain of Semliki Forest virus (SFV) induces rapid abortion following peripheral infection of 8-day pregnant mice (Atkins *et al.*, 1982; Milner & Marshall, 1984) but does not produce the range of effects characteristic of viruses such as rubella or bovine viral diarrhoea. The use of SFV (an alphavirus) as a model, rather than the flaviviruses previously used, has the advantages that its molecular biology is relatively well understood [early work has been reviewed by Kääriäinen & Söderlund (1978), and there have been many subsequent studies], it is easily manipulated in the laboratory, and it has been extensively used as a model for analysis of central nervous system infection (Atkins *et al.*, 1985). Here we report the isolation of four temperature-sensitive (*ts*) mutants of A7 SFV which differ from the wild-type in their effects on developing embryos. The mutants *ts*14 and *ts*22 in particular induce a range of developmental defects not characteristic of the wild-type virus.

## METHODS

*Mice.* The outbred Q/Fa strain of mouse was used in this study, rather than the BALB/c strain used previously (Atkins *et al.*, 1982), because this strain produces more embryos per mother and has been used in embryo culture experiments (Hearne *et al.*, 1986). Mice were housed at a temperature of 22 °C and subjected to 12 h cycles of dark and light. To produce mice of known gestation, groups of 12 virgin female and six male mice of age 8 to 12 weeks were placed together. The day on which a vaginal plug was found was taken to be day 1 of pregnancy. For infection experiments, pregnant mice were inoculated with $10^4$ p.f.u. of virus in 0·5 ml of phosphate-buffered saline (PBS), given intraperitoneally (i.p.) on day 8 of pregnancy, unless otherwise specified.

*Virus.* The methods of growing plaque-purified stocks of the A7 and neurovirulent L10 strains of SFV and of plaque assay using BHK-21 cells have been previously described (Barrett *et al.*, 1980). *Ts* mutants of the A7 strain were isolated following mutagenesis by *N*-nitro-*N*-methyl-*N'*-nitrosoguanidine and using the temperature shift technique previously described (Atkins *et al.*, 1974). The permissive and restrictive temperatures were 30 °C and 39 °C respectively. Stocks of plaque-purified *ts* mutants were grown in BHK-21 cells at 30 °C. Viral RNA synthesis and plating efficiency were measured as previously described (Atkins *et al.*, 1974; Atkins & Sheahan, 1982). Virus titrations of placental and foetal tissue were carried out using clarified 10% homogenates of pooled tissue from a single pregnant mouse (Atkins *et al.*, 1982). Extracts were stored at −70 °C prior to plaque assay.

*Embryo culture.* Embryos at the blastocyst stage were obtained on day 4 of pregnancy. Removal of the zona pellucida, culture to the egg-cylinder stage, and virus infection with $10^4$ p.f.u./embryo were carried out as described by Hearne *et al.* (1986).

In utero *survival.* Any mice not producing litters by day 21 of pregnancy were killed and their uteri stained by the method of Orsini (1962) to detect implantation sites. Abortion is defined as litter loss at any stage during gestation including cannibalism of litters by mothers immediately on delivery.

*Weight gain and postnatal immunity.* Litters surviving virus infection of the mother were reduced to six siblings on birth. They were then weighed on alternate days during a 19 day period. At day 19 after birth, the litters were challenged with $10^4$ p.f.u. of the L10 strain of SFV given i.p. in 0·02 ml of PBS. Statistical significance of weight gain results was tested as described previously (Barrett & Atkins, 1979).

*Viraemia.* Groups of non-pregnant mice were inoculated with $10^4$ p.f.u. of each mutant and A7, given i.p. in 0·5 ml of PBS. At daily intervals up to 6 days, two or three mice were sacrificed from each group and 300 µl blood samples were taken by cardiac puncture. These samples were stored in 2 ml of BHK medium (Gibco) at −70 °C for subsequent plaque assay.

## RESULTS

### *Isolation of mutants*

Initially, we attempted to isolate mutants by direct selection, an approach which has been successful for neurovirulence mutants (Barrett *et al.*, 1980). Two hundred plaque isolates from a mutagenized stock of SFV were injected into single 8-day pregnant mice. None of these mice produced litters, although control mock-infected mice did. The reason for the failure to isolate mutants is not clear at present. We therefore attempted to isolate *ts* mutants using the same mutagenesis treatment. As a preliminary step, we measured the body temperatures of duplicate pregnant and non-pregnant mice, both infected and uninfected, over a period of 3 days. Body temperatures were measured using a rectal thermometer at 6-hourly intervals. Temperatures were found to fluctuate between 36·6 °C and 39·4 °C with peaks during darkness and lows during light. Infection and/or pregnancy had no effect on body temperature. On the basis of these results, it was decided to set 39 °C as a restrictive temperature for the mutants, but to test their growth also at 37 °C. The permissive temperature for the mutants was 30 °C. After testing 100 plaque isolates, we isolated four *ts* mutants, numbered *ts*4, *ts*14, *ts*22 and *ts*26.

### In vitro *growth properties of* ts *mutants*

Table 1 shows the titres of working stocks of A7 and the four *ts* mutants at 30 °C, 37 °C and 39 °C. While the titre of A7 was reduced by less than 10-fold at 39 °C compared to that at 30 °C, that of the *ts* mutants was reduced by a factor of at least 200. The titre at 37 °C compared to 30 °C was reduced only for *ts*4. Viral RNA synthesis of A7 was reduced about 20-fold at 39 °C compared to 30 °C, whereas RNA synthesis by the *ts* mutants was reduced to virtually undetectable levels (Table 1). At 37 °C, only *ts*4 showed reduced RNA synthesis compared to 30 °C.

Similar results were obtained following infection of cultured early embryos (Table 1). Twenty-

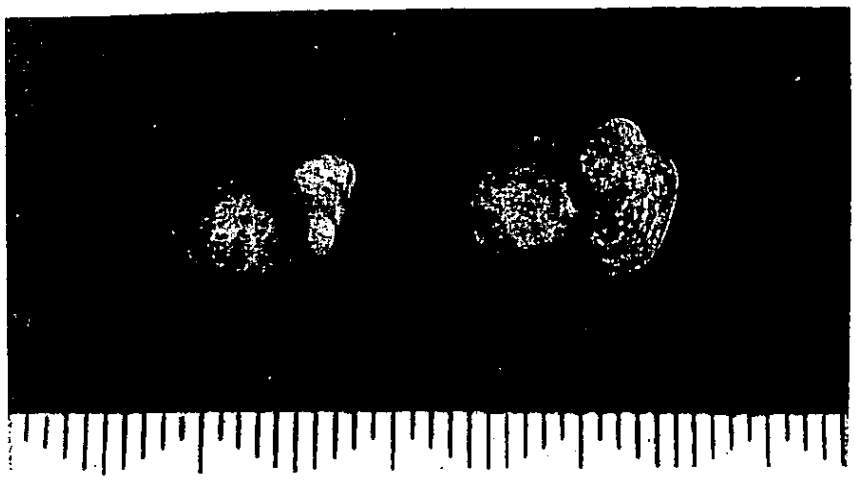

Fig. 1. Twelve-day embryos from an A7-infected mouse and a control mouse. Left, 12-day foetus and placenta from a mouse infected at day 8 of pregnancy with $10^4$ p.f.u. of A7 SFV given i.p. Right, foetus and placenta from a mock-infected mouse. The scale is in mm.

Table 1. *Growth characteristics of* ts *mutants in BHK cells and pre-implantation embryos in culture*

| Virus strain | Virus titre (p.f.u./ml) | | | RNA synthesis (c.p.m.)* | | | Virus yield (p.f.u./embryo)† | |
|---|---|---|---|---|---|---|---|---|
| | 30 °C | 37 °C | 39 °C | 30 °C | 37 °C | 39 °C | 37 °C | 39 °C |
| A7 | $2 \times 10^8$ | $1 \times 10^8$ | $6 \times 10^7$ | 4690 | 3600 | 217 | $7 \times 10^3$ | $3 \times 10^3$ |
| *ts*4 | $1 \times 10^8$ | $1 \times 10^4$ | $5 \times 10^2$ | 5887 | 845 | 23 | <5 | <5 |
| *ts*14 | $2 \times 10^9$ | $2 \times 10^9$ | $6 \times 10^6$ | 3913 | 3769 | U‡ | $7 \times 10^2$ | <5 |
| *ts*22 | $5 \times 10^9$ | $2 \times 10^9$ | $4 \times 10^5$ | 4872 | 5591 | 4 | $1 \times 10^4$ | 15 |
| *ts*26 | $2 \times 10^9$ | $1 \times 10^9$ | $1 \times 10^5$ | 4918 | 5239 | 12 | $2 \times 10^3$ | <5 |

* Cells were pulse-labelled with 1 μCi/ml [$^3$H]uridine at 6 to 8 h after infection, in the presence of 5 μg/ml actinomycin D, and the TCA-precipitable incorporation was determined. Incorporation by uninfected cells (100 to 300 c.p.m.) has been deducted. Each value is an average of three to six determinations.

† Blastocysts were infected with $10^4$ p.f.u. of virus and the yield produced at 24 h was assayed at 30 °C.

‡ U, Undetectable.

four h after infection with A7 at 39 °C, the yield was only slightly reduced compared to 37 °C, but the yield of the *ts* mutants was reduced by at least 140-fold and was only detectable for *ts*22. Again, *ts*4 was the only mutant which showed temperature sensitivity at 37 °C. These differences in virus yield were reflected in effects on embryonic development in culture; no effect could be detected following infection with *ts* mutants at 39 °C, or with *ts*4 at 37 °C. Embryos infected with A7 at 39 °C or with A7, *ts*14, *ts*22 and *ts*26 at 37 °C were necrotic by 48 h. Experiments involving embryonic infection at 30 °C were not carried out as uninfected embryos did not develop at this temperature. Note that *ts*4 was not a host-range mutant since it multiplied in cultured secondary mouse embryo fibroblasts and in mouse L cells at 30 °C (data not shown).

### *Effect of infection on embryonic development and postnatal survival*

Infection of mice with A7 on day 8 of pregnancy resulted in developmental arrest followed by abortion, usually on day 12. This effect is illustrated by Fig. 1. Infection with *ts*4 or *ts*26 had no effect on embryonic development (Table 2) or postnatal survival, and postnatal weight gain was comparable to uninfected controls. This was not the case for *ts*14 or *ts*22. Infection with these mutants produced a number of effects which were not observed for the wild-type virus or for uninfected controls. Fifteen out of 22 mothers infected with *ts*22 produced litters after the normal gestation period (19 to 20 days, Table 2). However, three of the litters containing live offspring also contained haemorrhagic dead foetuses (three to eight per litter). Four of these

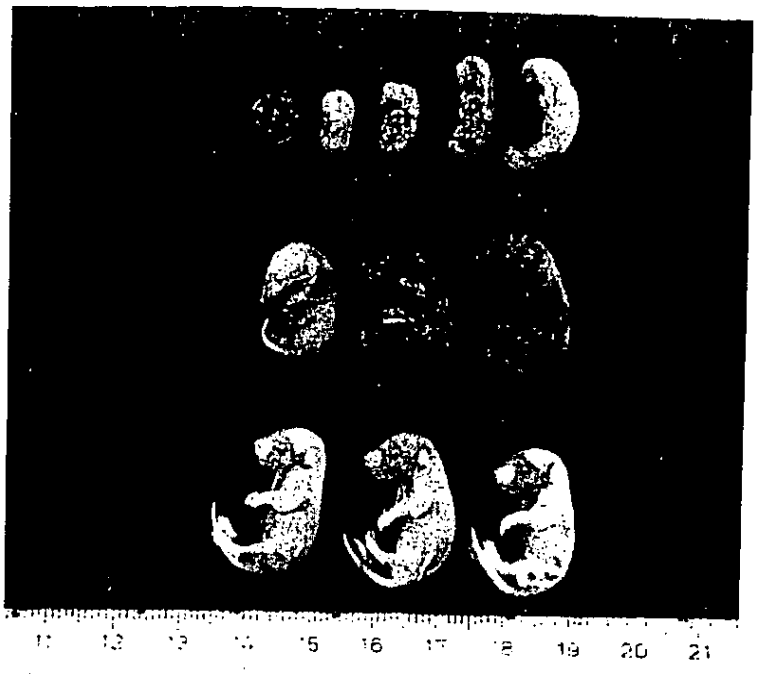

Fig. 2. Effects of infection with $10^4$ p.f.u. of *ts*22, given i.p. on day 8 of pregnancy, on embryonic development. Top row, four developmentally arrested foetuses at day 19 of pregnancy; the last on the right is a mummified foetus aborted on day 14. Middle row, haemorrhagic foetuses at day 19 of pregnancy. Bottom row, foetuses from mock-infected mice at day 19 of pregnancy. The scale is in cm.

Table 2. *Effect of infection with* ts *mutants on embryonic viability*

| Virus strain | Number of pregnant mice injected* | Number producing litters† | Number of litters surviving challenge‡ |
|---|---|---|---|
| A7 | 12 | 0 | NA§ |
| *ts*4 | 5 | 5 | 0 |
| *ts*14 | 7 | 5 | 5 |
| *ts*22 | 22 | 15 | 13 |
| *ts*26 | 6 | 6 | 3 |
| Uninfected | 8 | 8 | 0 |

* Mice were injected with $10^4$ p.f.u. of virus given i.p. on day 8 of pregnancy.

† Mice not producing litters by day 21 of pregnancy were killed and confirmed as having been pregnant by staining of uteri for implantation sites.

‡ Litters were given $10^4$ p.f.u. of the L10 strain of SFV i.p. on day 19 after birth.

§ NA, Not applicable.

mothers also ate one or more of their offspring during the postnatal period (up to 19 days after birth). Thirteen out of 15 of the surviving litters developed postnatal immunity, and weight gain for these litters was normal. Of those infected mothers which did not deliver, two carried haemorrhagic dead foetuses and one carried developmentally arrested foetuses to term. The remaining four mice aborted their litters on or after day 14 (compared to day 12 for the wild-type), and two of these aborted a mummified foetus each. With *ts*14, a minority (two of seven) of litters were aborted and all five remaining litters developed postnatal immunity. The initial experiments shown in Table 2 showed no apparent abnormalities among surviving litters. However, in a separate experiment with *ts*14, three mice infected at day 8 of pregnancy were killed on day 18 and the foetuses examined; all three showed the presence of haemorrhagic or developmentally retarded foetuses (5/12, 2/14, 5/10). In similar experiments for *ts*4 and *ts*26, no abnormal foetuses were found at day 18, but one litter containing 4/11 abnormal foetuses was

Table 3. *Effect of infection with A7,* ts*14 and* ts*22 on the viability of suckling mice*

| Virus strain* | Age of mice (days) | Number of survivors† | Mean time of death (days, ± standard error) |
|---|---|---|---|
| A7 | 3 | 0/5 | 2‡ |
| | 7 | 0/11 | 2·9 (± 0·1) |
| | 14 | 0/8 | 6·1 (± 0·1) |
| *ts*14 | 3 | 0/4 | 2‡ |
| | 7 | 0/12 | 4‡ |
| | 14 | 7/7 | NA§ |
| *ts*22 | 3 | 0/5 | 2‡ |
| | 7 | 4/13 | 10·5 (± 1·6) |
| | 14 | 6/10 | 11·8 (± 3·0) |

* Mice were given $10^4$ p.f.u. of virus i.p. in 0·02 ml of PBS.
† Out of total number injected.
‡ All infected mice died on day specified.
§ NA, Not applicable.

Table 4. *Induction of viraemia and growth of* ts *mutants in placental and foetal tissue*

| | Viraemia* | | | Multiplication in placenta† | | Multiplication in foetus† | |
|---|---|---|---|---|---|---|---|
| Virus strain | Peak titre (p.f.u./ml) | Time reached (days) | Time cleared (days) | Titre (p.f.u./ml) | Number of mice‡ | Titre (p.f.u./g) | Number of mice |
| A7 | $9 \times 10^5$ | 1–2 | 3 | $3 \times 10^8$ | 5/5 | $8 \times 10^{10}$ | 5/5 |
| *ts*4 | $<10$ | NA§ | NA§ | $<10$ | 0/3 | $<10$ | 0/3 |
| *ts*14 | $1 \times 10^3$ | 3 | 5 | $6 \times 10^6$ | 6/6 | $3 \times 10^4$ | 3/6 |
| *ts*22 | $1 \times 10^3$ | 2–4 | 5 | $3 \times 10^7$ | 3/5 | $3 \times 10^9$ | 3/5 |
| *ts*26 | $5 \times 10^3$ | 2–3 | 4 | $3 \times 10^8$ | 2/5 | $8 \times 10^2$ | 1/5 |

* Blood samples were taken from two or three non-pregnant mice at daily intervals up to 6 days after i.p. infection with $10^4$ p.f.u. Where one or more mice showed equivalent titres of virus on consecutive days, a range is given for the time the peak titre was reached.
† Placental and foetal tissue from single infected mice were pooled and assayed separately at day 16 of pregnancy following i.p. infection with $10^4$ p.f.u. on day 14 of pregnancy. The values given are the averages of positive samples.
‡ Number showing presence of virus out of number examined.
§ NA, Not applicable.

found for *ts*22. Examples of the range of effects on the developing embryo produced by *ts*22 infection are illustrated in Fig. 2.

The effect of i.p. injection of $10^4$ p.f.u. of A7, *ts*14 and *ts*22 on the survival of suckling mice was also tested (Table 3). All 3-day-old mice died regardless of the strain injected, as did 7- and 14-day-old mice injected with A7. However, a proportion of 7-day-old and 14-day-old mice infected with *ts*22 survived. Seven-day-old mice infected with *ts*14 died whereas 14-day-old mice survived.

### *Induction of viraemia*

The induction of viraemia by A7 and the four *ts* mutants was measured at daily intervals up to 6 days after i.p. injection of $10^4$ p.f.u. (Table 4). For *ts*4, no viraemia could be detected. However, for *ts*14, *ts*22 and *ts*26, a viraemia was produced but the peak titre was 100-fold lower than that produced by A7. Also, the time at which the peak was reached was about 1 day later and the duration 1 to 2 days longer for the *ts* mutants than for A7.

### *Virus multiplication in the placenta and foetus*

Assay of virus in foetal tissues was initially done by the method followed by Atkins *et al.* (1982). Fourteen-day pregnant mice were injected i.p. with $10^4$ p.f.u. and the foetuses removed were assayed for virus 2 days later. This was because sufficient foetal tissue was required for

Table 5. *Virus content of placenta and foetus of mice infected on day 8 of pregnancy**

| Mouse number | Mutant | Titre of placenta (p.f.u./g) | Titre of foetus (p.f.u./g) |
|---|---|---|---|
| 1 | *ts*14 | $1 \times 10^6$ | $2 \times 10^4$ |
| 2 | | $5 \times 10^6$ | $8 \times 10^6$ |
| 3 | | $3 \times 10^4$ | $1 \times 10^4$ |
| 4 | | $2 \times 10^6$ | $<10$ |
| 5 | | $2 \times 10^6$ | $<10$ |
| 6 | *ts*22 | $9 \times 10^5$ | $1 \times 10^9$ |
| 7 | | $6 \times 10^1$ | $<10$ |
| 8 | | $<10$ | $<10$ |
| 9 | | $6 \times 10^7$ | $1 \times 10^7$ |

* Mice were killed at day 18 to 20 of pregnancy. Placentas and foetuses from individual pregnant mice were pooled as a 10% (w/v) suspension.

virus assay before abortion occurred. The results obtained for *ts*14 and *ts*22 were unexpected. In three of six mice infected with *ts*14, virus was found in the placenta but not in the foetus (Table 4). In two of five mice infected with *ts*22, no virus was found either in the placenta or foetus. We therefore infected mice with *ts*14 and *ts*22 on day 8 of pregnancy and assayed the foetal tissue on days 18 to 20 (just before birth, Table 5). For *ts*14, virus was found in placental but not foetal tissue for two of five mice; for the remaining three mice, virus was found at the same or higher titres in placental compared to foetal tissues. *Ts*22 was detected in foetal tissue in two of four mice.

## DISCUSSION

We have isolated four *ts* mutants of the A7 strain of SFV which differ from the wild-type in their pathogenicity for developing mouse foetuses. One complication in the analysis of the effects of the mutants is the temperature sensitivity of the A7 strain, as originally reported by Woodward & Smith (1979). We have confirmed this and shown that the defect is expressed as a reduction in virus-specified RNA synthesis (Atkins, 1983; A. Hearne, unpublished results). It is clear that all four of our *ts* mutants have defects in RNA synthesis which are more extreme than the defect expressed by A7. This is consistent with the finding that 76% of *ts* mutants isolated for the closely related Sindbis virus showed less than 20% of the wild-type level of RNA synthesis (Atkins *et al.*, 1974).

Although 39 °C was a restrictive temperature for the *ts* mutants, three of the four mutants were able to multiply at 37 °C. Since the body temperature of the mice fluctuated between 37 °C and 39 °C, some multiplication of these mutants was possible in infected mice, as shown by the induction of viraemia and postnatal immunity in the offspring. The mutant *ts*4, however, was restricted at 37 °C as well as 39 °C, and so infection was abortive and no viraemia or postnatal immunity was induced.

Two of the mutants, *ts*14 and *ts*22, showed particularly interesting characteristics and were studied further. The modified pathogenicity of these mutants for foetuses correlated with modified virulence for suckling mice. The wild-type virus induces rapid foetal death and aborton in all infected pregnant mice at any stage of pregnancy except very early stages [< 5 days after conception (Atkins *et al.*, 1982; Milner & Marshall, 1984)]. In the majority of infected mice, *ts*14 infected the placenta more efficiently than the foetus. As well as abortion, *ts*14 and *ts*22 caused a range of developmental abnormalities in foetuses which were not found for the wild-type or the other mutants. These included developmental arrest, mummification, and possible teratogenic effects. Since both mutants were rapidly lethal for neonatal mice, the developmental defects produced may result from a partially effective immune response against foetal infection.

Use of the neurovirulence mutant M9 of SFV has provided us with a useful model to analyse central nervous system demyelination rather than the rapidly lethal encephalitis induced by the wild-type L10 strain (Atkins *et al.*, 1985; Atkins & Sheahan, 1982; Gates *et al.*, 1985). Similarly,

mutants *ts*14 and *ts*22 may provide useful models for the study of fundamental mechanisms in the production of virus-induced developmental defects, rather than the rapid abortion induced by the wild-type A7 strain.

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